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THE ETIOLOGICAL ROLE OF CARBON MONOXIDE IN

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THIS WORK IS DEDICATED TO MY PARENTS

AND DR. CARL A. NAU

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CHAPTER I

INTRODUCTION

Carbon monoxide (CO), an odorless, tasteless, colorless, non-irritating gas, is the most abundant and widely distributed air pollutant found in the lower atmosphere (Goldsmith, et al., 1968). It is an active reducing agent for chemicals at elevated temperatures, but it is principally encountered as a by-product of incomplete combustion of carbonaceous material.

Carbon monoxide as a component of the ambient air, has existed ever since the geologic ages prior to Pleistocene (Encyclopaedia Britannica, 1969). As a gas CO was discovered by Priestly in 1799 (Bour, et al., 1967) and the composition of CO was first established by Clement and Desormes in 1801. The Greeks knew of the effects of CO, and the Romans used it for suicides and punishment of criminals (Cooper, 1966).

There was relatively high concentrations of atmospheric CO from natural sources such as vegetative and animal growth as well as volcanic activities before man learned to make fire. At present, emission

of CO gas generally exceeds that of all other gaseous pollutants combined, especially in urban and industrial atmospheres.

Carbon monoxide is one of the most frequent forms of poisoning, second to only alcohol in the United States (Lilienthal, 1950a; Rose, 1969b). The results of extensive studies show that in excess of 3.5 billion tons of CO per year are produced in the Northern Hemisphere from natural sources, primarily from the oxidation of methane produced in swamps and paddies and the degradation of chlorophyll from rotting plants (Maugh, 1972).

According to these estimations, there are 530 million tons of CO present in the atmosphere. Only 102 million tons of this amount is released into the atmosphere annually by man-made sources (Swinnerton, et al., 1970).

It is obvious that man contributes a small percentage of CO to the atmosphere. The reports indicate that biological soil systems are an active and efficient natural sink for CO (Inman, et al., 1971). In natural processes, the soil system in a geographical area the size of the continental United States, can remove approximately 570 million tons of CO per year; an amount more than six times the estimated annual production of CO attributed to man's activities in America and over twice the worldwide production.

In 1968 it was estimated that 102 million tons of CO were released into the atmosphere by the major sources of emission (Air Quality Criteria for Carbon Monoxide, 1970). Over one-half of this quantity was produced by the gasoline-powered internal combustion engine. Specialized industrial processes accounted for approximately 10 percent of the total.

Even though natural soil systems for the removal of a significant amount of CO from the ambient atmosphere apparently exist, nevertheless, there is reason for concern. Industrialization with the dense population in the industrial centers and urbanization have increased tremendously. Many soil systems have been occupied and destroyed and can no longer act as active CO absorbers. At the same time, man-made CO emission has increased tremendously (Hoyt, 1967).

At the present time, emission of CO gas generally exceeds that of all other gaseous pollutants combined, especially in urban and industrial atmospheres. Large quantities of CO are emitted from petroleum refineries, iron foundries, kraft pulp mills, sintering mills, lampblack plants and formaldehyde manufacturers.

Major sources of CO production within the first three industries have been identified as arising from the cupola in the iron foundary, the catalytic cracking units in the petroleum refineries, the lime kilns, and the kraft recovery furnaces in the kraft paper mills. Besides all the major CO-producing industries, there are numerous operations such as automobile repair, traffic control, tunnel construction and arc welding, practices in which the occupational exposure to CO can be considerable. In fact, any industrial process or operation in which incomplete combustion of carbonaceous material is present may be of consequence as concerns occupational exposure to CO

Because of CO emission, this pollutant shows a pattern related to motor vehicle traffic flow. Also, the exposure to CO may vary by orders of magnitude with the highest exposure occurring when riding in the

car; thus setting up special dosage situations. Community sources seem to affect the background levels. There may be a relatively wider exposure of the general population to CO as the levels become higher over a large area.

In addition, there may be problems of shorter term exposure to higher levels of CO in commuter traffic. Most exposure to CO occurs during the air pollution episodes when CO levels appear to rise above the acceptable standard levels.

In Los Angeles, New York, Washington and other large cities, most of the ambient CO is emitted by transportation activities which generally are the source of 70 to 90%+ of the emitted CO. The combustion of fuels for the heating of occupied buildings and generating heat and power for industry accounts for a substantial portion of the remaining emissions (Ott, et al., 1967).

A Continuous Air Monitoring Program (CAMP) was begun in 1962, by the Division of Air Pollution to measure the concentrations of six gaseous pollutants, CO, NO, NO_2, SO_2, total hydrocarbons, and total oxidants in downtown areas of six cities: Chicago, Cincinnati, New Orleans, Philadelphia, San Francisco, and Washington. Concentrations for each of the six pollutants were obtained for 5-minute intervals. To obtain concentrations for longer time periods, all the available 5-minute values for the designated time period were averaged. In 1966, CO annual average concentrations measured at the CAMP sites in Chicago, had an arithmetic mean value of 12.5 ppm for the year.

A more meaningful measure is the frequency distribution of 8-hour concentrations. This has been done by members of National Air Pollution

Control Administration. The maximum 8-hour average concentration measured for Chicago for the period of December 1, 1961, to December 1, 1967, was 44 ppm, and the minimum was zero. Concentrations of carbon monoxide which exceed 12 ppm for a period of 8 hours or more would tend to raise the carboxyhemoglobin level by 2%. Data are available for the other cities for differing time periods.

The levels of exposure to CO for commuters during rush hours were also determined. National Air Pollution Control Administration's Division of Air Quality and Emission Studies found that during a half-hour trip, averages of CO were discovered to be in the 10-40 ppm range in the 15 cities, with short-term peaks as well over 100 ppm in the larger cities. The levels varied significantly by type of route also. Average over all 15 cities, trips confined to center cities showed levels of 31.5 ppm compared to 21.6 ppm on arterial streets leading to downtown and 21.1 ppm on expressways. These values changed noticeably during the air pollution buildup or episodes. The reports show that on November 24, in New York, the peak value was 35 ppm, whereas two days earlier, it was 8 ppm.

Most epidemiologic studies of CO health effects have dealt with occupationally exposed groups. The effects usually observed are increased levels of carbon monoxide saturated hemoglobin, carboxyhemoglobin (COHb), and the disputed "chronic carbon monoxide poisoning syndrome" (Sievers, 1942; Grut, 1959). Grut studied 721 men of whom 46% had chronic CO poisoning. This syndrome was characterized by fatigue, headaches, irritability, dizziness, and disturbed sleep. Some subjects were noted to

have abnormal neurologic problems. Vehicle inspectors exposed daily to CO concentrations ranging from 10 to 150 ppm were studied. Differences in COHb levels between exposed and non-exposed groups were only significant in nonsmokers, (Hofreuter, et al., 1962). Blood samples were also obtained from policemen and drivers in Rotterdam and Amsterdam before and after work. Increased COHb levels were demonstrated in these occupationally exposed subjects. A control group exhibited no such increase (de Buirn, 1967).

Studies to determine the levels of COHb in blood samples of 331 traffic policemen in Paris showed that increase of COHb of nonsmokers and showed very little increase in COHb of smokers after a five hour work period. Cigarette smokers did not smoke while at work (Chovin, 1967).

The effects of CO as an air pollutant on human health have been pondered for years and much has been written about the amount of this gas discharge into the atmosphere and its potential effects on life. However, it was not until 1958, that attempts were made to test the hypothesis that during high periods of CO pollution, individuals hospitalized with acute cardiovascular disease were adversely affected in 35 Los Angeles County hospitals (Cohen, et al., 1958). Three years later, Hechter compared air pollution with daily mortality and concluded that there was a possible correlation between daily highs and mortality and postulated that this was seasonal in nature (Hechter, et al., 1961).

The major concern in CO as an air pollutant are the additions of the automobile exhaust control legislation to the Clean Air Act by the 89th Congress (Giever, 1967), the formation of the Laboratory of Medical

and Biological Sciences in the Division of Air Pollution and the inception of the Fried Laboratory (Hueter, 1966a; Goldberg, et al., 1967b) which selected CO as the primary contaminant to be studied by behavioral methods.

Further evidence of the significance of chronic CO as a health hazard may be indicated by the action of the Committee of Threshold Limits of the American Conference of Governmental Industrial Hygienists, which in 1964, recommended that the Threshold Limit Value (TLV) for CO in workroom air be lowered to 50 ppm by volume; from the figure of 100 ppm which it had sustained for many years (Giever, 1967).

The California State Board of Public Health in 1959, adopted standards for ambient air quality, and stipulated as a "serious level" 30 ppm of CO for 8 hours or 120 ppm for 1 hour.

Cohen and his associates studied the effect of CO pollution in the Los Angeles Basin on case fatality rates of patients with myocardial infarction. These studies involved hospitals where it was found that there was an increased myocardial infarction-case-fatality-rate in areas of high levels of CO pollution (Cohen, 1969). By using a complicated mathematical model involving complex regression analysis, Hexter, et al., 1971, found a statistical significance ($p < .002$) between CO levels and mortality in the Los Angeles Basin.

Most exposures to CO, other than the low levels involved with air pollution, are occupational and are under regulated and controllable situations (Air Quality Criteria of Carbon Monoxide, 1970). The most significant exception to this is the exposure to low levels of CO for long periods

of time, ranging from a month to a lifetime, and in the range of CO concentrations that would produce 0.5-10% COHb. Also, in the case of occupational exposures to CO, a worker's smoking habits must be taken into consideration (DuBois, 1969). This factor has not been generally considered until the past few years.

These recent reports raised many questions concerning the effects of low-level exposures to CO for a long period of time. The diversity of opinions and the conflicting experimental evidence existing in this area; does not permit the clear-cut assessment of the scientific merit of such data or its extra pollution to the normal working population. If reliable data became available which clearly demonstrated impairment of employees' health during exposure to very low levels of CO, then the criteria for the recommended standard would be reversed on the basis of the additional evidence.

There are clear evidences from the intensive studies that effects of exposure to the concentrations of CO found in cigarette smoke is much more hazardous to human life than had been realized in the past (Ringold, et al., 1962). A level of 5.9% COHb was found to be the median value in moderate cigarette smokers who inhale. Relatively low levels were found in pipe smokers who also inhaled. COHb levels by analysis of expired air were estimated for 299 post office workers and gave similar results (Goldsmith, et al., 1963a; Coburn, et al., 1965b; Lawther, 1971c; Cohen, et al., 1971d).

Exposure for five hours to between 10 to 12 ppm of CO has been shown to increase the COHb levels in non-smokers by at least 0.5%. Such an increase adds appreciably to the body burden of COHb in those who do

not already have such a body burden from cigarette smoking. Longer exposures could have produced a somewhat greater increase.

There are clear evidences from the intensive studies that the effect of exposure to the concentrations of CO found in cigarette smoke may be causing emphysema which is a disease occurring with increasing rapidity among heavy cigarette smokers. Emphysema and lung cancer have much in common as to cause change in the bronchial mucasal epithelium--surface cells of the air passages of the lungs (Von Oettingen, 1944). Carbon monoxide in cigarette smoke produces arterial changes that help to cause damage to the air sac walls. This literature contains significant scientific information on the pollutant gas (CO) relative to its effect on the hardening of the arteries (arteriosclerosis) and damaged heart muscle (Astrup, et al., 1970a; Bartlett, 1968b). This leads to myocardial infarction which if extensive enough, causes death. The reports as a whole reflect a consistent pattern, namely cigarette smoking imparts profound alterations in the human coronary system as a result of myocardial fiber hypoxia.

This particular air pollutant (CO) combines with significant quantity of hemoglobin and myoglobin causing a reduction in operation efficiency. It is reasonable to assume that the compensatory mechanisms are not sufficiently sensitive to prevent damage to myocardial fibers or that there is not enough oxygen available to prevent the damage regardless of the amount of blood flow through the coronary system (Myasnikov, 1958).

These damages are usually reported on autopsies as myocardial necrosis. In an average normal man (standard man, 70 kgm), myocardial tissue

comprises 0.5 percent of the total body and consumes oxygen (O_2) at the rate of 9.5-10 ml/100 gm/minute. Blood flows to the myocardium at the rate of 84 ml/100 gm/min. which is a rather small quantity in comparison with other organs (Ganong, et al., 1971a; Taylor, 1965b). Based on this comparison, it is evident that the myocardium needs the largest quantity of O_2 and expels greatest amounts of arteriovenous O_2 difference. The myocardium consumes 11.6 percent of the O_2 while that consumed by the total body is only 4.7 percent of the total cardiac out-put.

The average smoker maintains a COHb saturation of 5 to 8 percent constant which later becomes critical in the circulation (Clayton, 1968a; Bartlette, 1968b). The body stores CO not only by combining it with hemoglobin, but also extravascularly stores it bound to myoglobin. The cytochromes also catalyze it with the use of peroxidases. There is now increasing evidence that the myoglobin of heavy smokers may be saturated with carboxyhemoglobin (Coburn, et al., 1963a; Coburn, 1970b). The potential significance of this is that myoglobin is the mechanism for O_2 transport in the tissues and therefore the major source of oxygen for the myocardial tissue. Similar carboxyhemoglobin levels have been shown to intensify myocardial ischemia and to enhance development of arrhythmia during exercise in subjects older than 40 years (Krelson, 1972), indicating that coronary obliterations increase the susceptibility of carbon monoxide exposure (Gray, 1966).

Electrocardiographic changes are often found, but occasionally the electrocardiogram does not show any abnormality. The precipitating factors of coronary insufficiency pattern might be shock or right ventricular dilatation.

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Some investigators suggest that adequate functioning of the heart increases the general risk in CO poisoning (Lewey, et al., 1944). Studies with dogs exposed to 100 ppm for eleven weeks, indicated that significant changes in (EKG) electrocardiographic pattern occurred.

Under hypoxic conditions increased oxygen supply to the peripheral tissues can be accommodated by increased blood flow (via vascular dilatation) and increased oxygen extraction by the tissues. The myocardium under these circumstances appears to increase flow of blood rather than to extract an additional amount of oxygen from the coronary circulation. While the peripheral tissues normally extract only 25 percent of the oxygen content of perfusing arterial blood during resting conditions, the myocardium extracts 75 percent, thus leaving the mixed venous blood only 25 percent saturated (Jones, et al., 1965). This mechanism has the over-all effect of maintaining the myocardial oxygen tension at a higher level which would be present in other muscle tissue and thus increase a continual aerobic metabolism, even under hypoxic duress. In terms of oxygen tension, the mixed venous blood of the peripheral tissues is approximately 40 mm Hg while the mixed venous blood of the coronary circulation is only 20 mm Hg. (In the presence of COHb, the oxyhemoglobin dissociation curve shifts to the left.) However, the arteriovenous difference can only be maintained by an increased flow in the coronary circulation (Niden, et al., 1956). Their results show that, in an individual with diminished coronary circulation, because of coronary heart disease, a decrease in mixed venous oxygen tension of the myocardium precipitated by an inability to maintain the normal arterio-

venous gradient. This hypoxic effect is further enhanced by an increase in cardiac rate and out-put as a general response to peripheral tissue hypoxemia. In this situation, a person with diminished coronary circulation caused by coronary heart disease, consequently may be constantly near the point of myocardial tissue hypoxia (Ayres, et al., 1969).

The study of the relationship between CO poisoning and an elevated titer of serum lactic dehydrogenase as an indicator of myocardial damage (Jaffe, 1968) indicated that even "normal" amounts of carbon monoxide may play an important role in the coronary attacks. Other studies observed a statistically significant aberration in the plasma lactic dehydrogenase isoenzyme distribution which was considered to be highly indicative of myocardial tissue damage (Lassiter, et al., 1972).

Another study of low level CO exposure to the percentage of patients who have coronary heart disease with angina pectoris showed that a small additional decrease in the oxygen saturation of blood brought about by mild exercise might be feasable. The degree of blood oxygen desaturation demanded with 10 percent COHb loading is rather severe (Dinman, 1968).

In a study of normal young males and clinically normal middle aged males, low level CO with a treadmill exercise showed statistical differences in the electrocardiogram (ECG) patterns. The investigators suggested that the low-level CO exposure may augment the production of exercise-induced myocardial ischemia in persons with preexisting subclinical heart disease, contribute to the development of myocardial dysfunction, and increase the arrhythmias (Anderson, et al., 1971).

An interesting observation from the study of sheep in altitudes of 7000 meters presented significant evidence that oxygen breathing permits the heart to do more work and reduces the amount of oxyhemoglobin causing the drastic change in brain and cardiovascular system and clotting of the blood (Cheymol, et al., 1955). Important implications of a study of the effect of CO to the muscle fatigue and impairment of O_2 transport to the tissues is made clear. Delayed recovery from CO induced fatigue suggests intracellular binding of CO as mentioned above producing hypoxia. The affinity of CO for myoglobin and cytochrome oxidase may exceed that of hemoglobin. The binding of CO at either site could decrease energy production, and in turn, the force of muscle fatigue (Petajan, et al., 1973).

It has been stated that enzymatic oxidations are sensitive to the traces of the inhibitors which are known to inhibit radical reaction chains, e.g., malonic acids, organic iodides, sulfites, cyanides, and CO (Mahler, et al., 1966). Furthermore, CO is known to inhibit some respiratory enzymes which contain iron or copper atoms as an essential part of their catalytic mechanism. Carbon monoxide is also known to react with microsome B_{420}, perhaps the terminal oxidase for microsomal electron transport with either cytochrome b_5 (liver mitochondrial microsome) or microsomal hydroxylase functioning as an electron donor (Mahler, Ibid).

It has been shown that chronic CO intoxication in rats resulted in a decrease of activity in cholinesterase in blood serum, which can be used as an index for the degree of intoxication, and that changes in the organism under chronic exposure to CO are persistent, as demonstrated by the

incomplete restoration of the activity of cholinesterase for three months after the inspiration of CO is stopped.

Studies of enzyme changes from exposure to low level CO (Rozera, et al., 1959) showed a decrease in erythrocytic phosphatase activity, and with continued exposure, a less decrease in erythrocyte alkaline phosphatase and serum phosphatase. Rabbits were used to show the effect of acute CO poisoning on riboflavin level in blood and tissues. It was observed that riboflavin was released to the blood via the muscles probably from protein decomposition, and that the differences between exposed and controlled groups were statistically significant (Jerzykowski, et al., 1963).

Several specific alterations in the number of endocrine organs have been attributed to CO. Several cases of acute hyperthyroidism following exposure to CO and increased activity of the thyroid and decrease of tyrotropic hormone is evidenced by anatomical changes (Lilienthal, 1950). It is found that repeated exposure to CO causes inhibition of gastrointestinal mobility (Patterson, 1938).

An interesting study approached the conclusion that there is a correlation between CO level and automobile accidents. A statistical and epidemiological strategy for this purpose has been developed and utilized (Ury, 1968) for studying the association of oxidant and automobile accidents. Neurophysiologic and behavioral effects of low-level to moderate CO exposures, were extensively reported.

These significant findings suggest deep concern for occupational

safety and health considerations. Several studies have been reported concerning searches for the effect of low-levels of CO using objective electrophysiological measures as spontaneous electroencephalograms (EEG), visual evoke responses (VER) sleep patterns, and conditioned electrocortical reflexes (Grudzinska, et al., 1963).

Several investigators have reported the effect of low-level CO exposure on the central nervous system (CNS) and changes in behavioral patterns (Chappell, et al., 1967) on rats. In general, increasing COHb levels result in corresponding depression of the CNS showing a progression from slight headaches at 10-20 percent COHb, coma with intermittent convulsions at 50-60 percent, and death at 70 to 80 percent COHb. In the case of subclinical effects of low level CO exposure on CNS these are less well documented. The reason for this is that the current physiological and toxicological methods are inadequately analyzing the subtle effects of chronic environmental pollutants (Beard, et al., 1967). Chronic contamination of the environment by various chemicals, gases, heat, radioactive materials, noise, solid waste, sewage, and trace metals throughout the world has opened up many new areas of research. The ultimate aim of research and toxicologic studies is to determine the effects of pollutants on both human health and welfare. To obtain this it is essential to conduct animal studies to evaluate the potential toxicity of different pollutants.

As mentioned earlier, CO is considered one of the most important and widely distributed urban atmospheric pollutants and reportedly constitutes a health hazard. The fact that so few investigations have been concerned with this gas and its effect on fibrinolytic activity and coronary

thrombosis explains the failure of a literature search to reveal any other pertinent studies relevant to the etiological role of CO in fibrinolytic activities in the blood and coronary thrombosis. There have been no systematic investigations or careful accumulation of information in these aspects of CO poisoning.

Purpose and Scope

The purpose of the present investigation was to accumulate, study, elucidate, and amplify data concerning the effects of exposure of rabbits to low concentrations of CO on fibrinolytic activity.

In an effort to study the abnormalities in the blood clotting mechanism of low-level CO, rabbits were exposed to continuous 50 ppm CO for a period of 8 weeks. Blood samples were drawn twice a week for hemoglobin, carboxyhemoglobin, oxyhemoglobin, and blood fibrinolytic activity which was measured by whole blood clot lysis, a modified Fearnley technique, euglubulin lysis, fibrin plate, caseinolytic assay, fibrinolysis, serum fibrin/fibrinogen degradation products. Vascular and myocardial tissues changes were studied by microscope. Control and experimental rabbits were handled alike as far as possible, except that the experimental group was exposed to controlled amounts of CO. Urine was collected and studied for the fibrinolytic activity in rabbits of both the test and control groups. Carbon monoxide was measured with a Beckman infrared gasanalyzer. CO-oximeter and other advanced techniques were used for the measurement of oxyhemoglobin, hemoglobin, and carboxyhemoglobin.

A statistical model was developed to implement and expedite analysis

of small changes in large amounts of data. The data were subjected to analysis of variance, and the various treatment groups were compared by using a t-test and Duncan's Multiple Range Test. A 0.05 statistical level of significance was chosen for this study.

In order to make sure that the elevated fibrinolytic activity was due to the plasminogen activator that was released from the endothelial cells of the vessel walls, a third group of 6 rabbits were maintained under the same environmental condition in chambers. This time, concentration of the CO was increased to 300 ppm/8 hrs. for 5 days a week. A group of 3 rabbits were placed in one chamber and another group of 3 rabbits were placed in the other chamber. Both groups were then given the same concentration of CO (300 ppm/8 hrs) for 4 weeks. Concurrently a group of three rabbits were given epsilon-aminocaproic acid, (EACA, 200 mg/Kg) a known inhibitor of fibrinolysis (through inhibition of the activation of plasminogen).

Histological and microscopic examinations were done on all the experimental animals' myocardial and arterial tissues.

CHAPTER II

LITERATURE REVIEW

The hemoglobin molecule plays an important role in oxygenation and deoxygenation of the blood. In order to understand the chemical asphyxia and physico-chemical changes in the body tissues it is necessary to understand the physico-chemical behavior ofahemoglobin molecule as well as its relation to O_2 and CO gases.

Physico-chemical Behavior of Hemoglobin

Hemoglobin is a conjugated protein consisting of an iron-containing porphyrin ring, heme, and a water soluble protein, globin. The molecule is composed of four hemoglobin chains. Each hemoglobin chain is composed of a globin moiety and a heme moiety. The globin moiety is a protein made up of over 140 amino acids.

There are four common sequences, and it is suggested that (Ingram, 1963) all these globin chains probably had a common evolutionary origin. The heme moieties are always the same and have similar molecular structures. The heme molecule is planar, its iron being attached to the proximal and distal histidyl residues of a globin chain. Upon oxygenation, the oxygen (O_2) slips between the Fe and the distal histidyle residues. The amino acid sequence only indicates the primary structure of the hemoglobin chain The protein has spring like α-helical regions which give the secondary

structure. Additional attractive forces twist the molecule into the worm-like tetramer formation.

Adult hemoglobin is formed when an α-chain (globin plus heme) and a β-chain associate to form a dimer and then two dimers associate to form a tetramer of the composition $\alpha_2 \beta_2$. The four subunits are irregular in shape, but rest together very tightly. The four hemes (iron plus porphyrin) lie semi-buried in non-polar crevices in their individual globins; water filling a cavity in the middle of the entire hemoglobin molecule.

The role of the hemoglobin molecule is the transport of oxygen, which it performs by virtue of its ability to combine with oxygen. The capacity to combine reversibly with molecules of oxygen without undergoing any demonstrable gross chemical change is the basis for its physiologic function (Schejter, et al., 1963). The remarkable progress in defining both the chemical and stearic configuration of this molecule has allowed some tentative exploration of those chemical and physical factors which contribute to its function. Similar heme-containing chromogen molecules, including the cytochromes and myoglobin, are widespread throughout the cells of the body. None of these has the characteristics of hemoglobin which, by virtue of its sigmoid-shaped oxygen dissociation curve, accepts molecular oxygen at relatively high tensions in the lungs and releases it to the tissues at relatively low tensions.

The evolutionary process apparently reached a functional compromise in the structure of the hemoglobins because these molecules exist in physiological conditions as aggregates of monomeric hemoprotein chains. On the basis of this structure, several thermodynamic situations can cause

the sigmoid shape of the titration curves of the hemoglobin-oxygen equilibria. Of these most widely discussed are those defined as intra molecular and intermolecular interactions. The hypothesis of intramolecular interactions, which considers tetrameric hemoglobin as the only molecular species participating in the equilibrium, was first formulated by Adair (1925). This hypothesis implies that the hemes of a single molecule are not independent of each other in their affinity for oxygen. The mathematical consequences of such a situation have been exhaustively analyzed by Wyman (1964) and its physical meaning is contained in the definition of free energy of intramolecular interaction. Very accurate measurements, especially at low and high percentages of saturations, permitted the estimation of the microscopic association constants defined by Adair.

The concentration effect has been interpreted by Roughton (1965) as due to van der Waals interactions between adjacent tetrameric hemoglobin molecules. The fundamental issue here is not, however, the nature of the forces involved in these interactions. The problem to be faced is whether it is correct to disregard the concentration effects in writing the equations that describe the hemoglobin-oxygen or carbon monoxide equilibrium. Actually, the existence of concentration effects can be taken as evidence that the intermolecular interaction hypothesis is, at least to a certain extent, correct; concentration effects on the position of the equilibrium occur only in the reaction where there is a change in the number of molecules. There are cases for which the generalized stoichiometric equation (Schejter, et al., 1963) applies almost quantitatively.

The kinetic model for the reaction of oxygen with hemoglobin is assumed to be expressed by

$$Hb + O_2 \underset{k}{\overset{k'}{\rightleftharpoons}} HbO_2$$

There are several reasons for selecting this simplified overall approach. It has been shown (Gibson, 1959a; Gibson, 1959b; Moll, 1969c) to hold empirically for the overall reaction and to be a good approximation to the Adair Intermediate Compound Hypothesis (Gibson, 1959) which is the generally accepted model for the oxygenation of the hemoglobin molecule.

The Adair kinetic model for the reaction of oxygen or CO with hemoglobin is completed four successive reactions (Fleischmen, 1968).

$k_1'/k_1 = K_1$ $\qquad Hb_4 + O_2 \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} Hb_4O_2$

$k_2'/k_2 = K_2$ $\qquad Hb_4O_2 + O_2 \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} Hb_4O_4$

$k_3'/k_3 = K_3$ $\qquad Hb_4O_4 + O_2 \underset{k_3}{\overset{k_3'}{\rightleftharpoons}} Hb_4O_6$

$k_4'/k_4 = K_4$ $\qquad Hb_4O_6 + O_2 \underset{k_4}{\overset{k_4'}{\rightleftharpoons}} Hb_4O_8$

Similar reactions for carbon monoxide:

$1_1'/1_1 = L_1$ $\qquad Hb_4 + CO \underset{1_1}{\overset{1_1'}{\rightleftharpoons}} Hb_4CO$

$1_2'/1_2 = L_2$ $\qquad Hb_4CO + CO \underset{1_2}{\overset{1_2'}{\rightleftharpoons}} Hb_4(CO)_2$

$1_3'/1_3 = L_3$ $\qquad Hb_4(CO)_2 + CO \underset{1_3}{\overset{1_3'}{\rightleftharpoons}} Hb_4(CO)_3$

$1_4'/1_4 = L_4$ $\qquad Hb_4(CO)_3 + CO \underset{1_4}{\overset{1_4'}{\rightleftharpoons}} Hb_4(CO)_4$

The intermediate compounds of the reactions have not been clearly detected, but its clearly understood that hemoglobin mainly exists as either the completely unsaturated Hb_4 or completely saturated Hb_4O_8 state (Gibson, 1959).

The forward and reverse rate "constants" k' and k are actually not constants because of the accelerated oxygenation process resulting from the heme-heme interactions. In these four reactions k' is a function of all four of the intermediate (k')'s of the Adair model and it varies directly with saturation due to increased importance of the k_4', the fourth rate constant of the Adair model.

As a given saturation, k' and k are constants, and their ratio, $K = k'/k$, represents the equilibrium constant for that saturation. However, as the saturation increases, the equilibrium "constant," K, increases too.

This K value can be calculated from the oxygen dissociation curve. The conditions from chemical equilibrium in whole blood between oxygen and hemoglobin are usually expressed in terms of the dissociation curve. The dissociation curve gives the equilibrium relationship between the

partial pressure of oxygen in the plasma and percent saturation of the hemoglobin in the red cells (Mochizuki, 1958).

The saturation refers to the fraction of the hemoglobin that has been oxygenated to form oxyhemoglobin. Since the solubility of oxygen in the red cells is known (Sendroy, et al., 1934) for a given partial pressure of oxygen in the plasma, it is possible to find the concentration of dissolved oxygen in the cell which is in chemical equilibrium with hemoglobin of a given saturation. This dissociation curve is a function of temperature and pH and has been reported (Karhan, 1968) at 25°C for pH values of 8.6 and 8.1 respectively.

In the kinetics of hemoglobin reaction, it is important to consider not only the rates for uptake and release of the iron ligand but also the rate of any ligand-link process, such as the Bohn effect, the effect of organic phosphates of ions of carbon dioxide in ligand association and dissociation processes.

In the processes of kinetics, effects of ligand linked association and dissociation of the hemoglobin tetramer, $\alpha_2\beta_2$, may reversibly dissociate into subunits of lower molecular weight $\alpha\beta$ dimers and single chain molecules. These association and dissociation equilibria may be ligand linked. These effects are due to association and dissociation processes expected to show up when kinetic experiments are performed under conditions which favor dissociation (Churchill, 1960).

There are major factors which affect the rate at which red cells can take up oxygen and CO. Carbon monoxide combines with hemoglobin at the same site as does oxygen, both ligands attaching to the iron atom of the porphyrin group. The slow rate at which CO dissociates from carboxy-

hemoglobin causes it to have an affinity for hemoglobin approximately two hundred fifty times as great as the affinity of oxygen. This enables CO in small concentrations to displace oxygen from hemoglobin thus reducing the oxygen carrying capacity of the blood, and is largely responsible for the toxicity of carbon monoxide.

Carbon monoxide binding tightly to hemoglobin produces hypoxemia in the short period of exposure. Another property of CO is that it combines with hemoglobin less rapidly than does oxygen. This enables one to measure some of the CO reaction rates more easily and accurately than the corresponding oxygen reaction rates (Roughton, 1957). The gas uptake by the red cells can be explained briefly by reaction rates of the carbon monoxide and oxygen with hemoglobin. The methods for measuring the reaction rates published by Hartridge and Roughton (1923) and much of the present knowledge is due to the intensive work of Roughton and his coworkers.

The velocity constants for the four successive stages of oxygenation of the hemoglobin tetramer are given by the symbols k_1', k_2', k_3', k_4' and the constants for the deoxygenation of the tetramer by k_4, k_3, k_2 and k_1. The use of prime (') indicates association velocity constant and it's absence indicates a dissociation velocity constant. For the reaction of CO with hemoglobin l is used instead of k. In cases where the overall rate of gas combination with reduced hemoglobin is measured, the symbol k' or l' is used without a subscript. To describe the rate at which CO replaces oxygen from the fully saturated tetramer, Roughton, et al. (1945) introduced the symbol m' defined by equation:

$$\frac{d\,[COHb]}{dt} = m' \frac{[CO]\,[O_2Hb]}{[O_2]}$$

The value of m' is not constant but varies with the ratio CO / O_2 and thus is referred to as a pseudonstant rather than as a constant. Its relation to the more fundamental constants is expressed by the equation:

$$m' = \frac{k_4 l_4}{4k'_4\{1 + \frac{1'_4\ CO}{k'_4\ O_2}\}}$$

Roughton, Forster and Canden introduced the term m'_∞ to describe the rate of the replacement reaction when the ratio CO / O_2 diffusing capacity is measured with low concentrations of CO. From the previous equation it follows that

$$m'_\infty = \frac{k_4 l'_4}{4k'_4}$$

The symbols of l' and k'_4 without the subscript "c" refer to reactions of hemoglobin in solutions. In describing the velocities of uptake of oxygen and CO by reduced red cells the symbols k'_c and l'_c are used and m'_c is used for the rate of CO replacing O_2 in cells.

The definition of k'_c is $HbO_2 \overset{k'_c}{\rightleftharpoons} Hb + O_2$

$$\frac{d[O_2Hb]}{dt} = k'_c\ [O_2][Hb]$$

O_2 being the concentration of oxygen in the fluid immediately surrounding the red cells, and the reverse reaction again being neglected. Similar equation apply for l'_c and m'_c.

The use of expressions for the apparent velocity constants for gas uptake by cells has certain limitations. The rate must be measured

as soon after the reactants are mixed as possible. This is because the equations of Roughton describing the combined processes of diffusion and chemical reaction into the cells are not valid except at zero time (Roughton, et al., 1957). The reverse reaction must also be neglected in order to solve the equations, the error introduced by neglecting it is minimized by measuring the rate at zero time and, in the case of CO, by the inherent slowness of the dissociation reaction. In the calculations all the combination velocity constants are second order and expressed in units of "liters per millimole per second" which is equivalent to "per millimolar per sec" abbreviated as $mM^{-1}sec^{-1}$. The dissociation velocity constants and m', m'_∞ and m'_c are first order and are expressed in units of sec^{-1}.

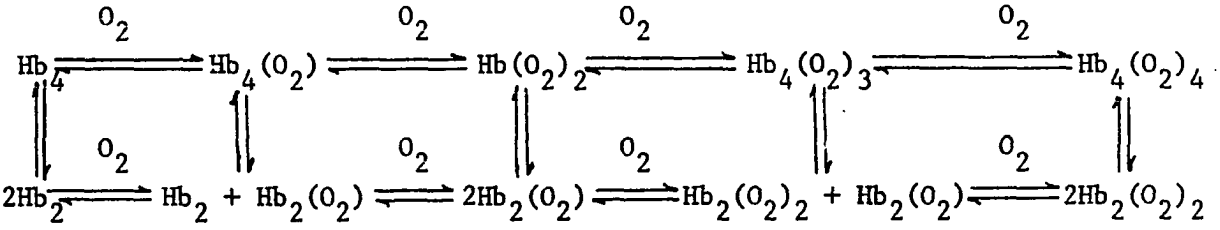
The fact that the sedimentation constants of hemoglobin and oxyhemoglobin at physiological concentrations approach the values expected for a tetramer, seemingly militates against the existence of different intramolecular interactions, or affinities, in the two states. Nevertheless, a number of observations indicated that dimers do exist even at large concentrations (Michaelis, et al., 1938).

Thus measurements of escape time through membranes indicate appreciable deaggregation of carboxyhemoglobin at concentrations of 4mg/100ml (Guidotti, et al., 1963). Another fact that is in keeping with intramolecular interaction hypothesis, while in open contradiction with the intramolecular interaction hypothesis, is the effect of salt concentration on the oxygenation curves (Barcroft, 1928a; Rossi-Fanelli, et al., 1961b).

In this case the direct relationship between the shape of the titration curves and the extent of deaggregation is clear. Molecular weight decreases, while the interactions increase, at higher salt concentrations

(Rossi-Fanelli, et al., 1961). Some investigators pointed out that this result is paradoxical in terms of the intramolecular interaction hypothesis (Antonini, 1965).

It is possible to envisage a compromise between both theories and the experimental facts, by assuming that the partially oxygenated intermediates are in equilibrium with their deaggregation products. This would lead to a system of consecutive boxes:



This complicated scheme does not include all the possible intermediates. Distinguishable dimeric species may occur, due to the different structure of the α and β chains. The hypothesis of consecutive boxes does not require appreciable values for the degredation constants of all the tetramene intermediates. In fact, if only one of these intermediates splits into dimers the oxygenation curve will be sigmoid in shape.

In this sytem, free energies of deaggregation play the role reserved for the free energies of intramolecular interaction in Adair's hypothesis. If in addition, the latter have significant values, there will be a cooperative effect between both types of interactions.

It also appears that the effect of high salt concentration is greater on the aggregation constants than on the intramolecular interactions. Undoubtedly the incorporation of aggregation constants complicates the estimation of thermodynamic parameters; at the same time, it takes into account concentration effects in agreement with the observed facts.

The equations that will finally describe such a system will also permit quantitative prediction of the concentration effects and will allow estimation of the true magnitude of the intramolecular interactions. The system of consecutive boxes also suggests an explanation for the striking dependence of viscosity and dielectric increment of hemoglobin solutions on the degree of oxygenation. These properties do not vary steadily, but instead they increase and decrease twice, showing maxima at about 25% and 75% saturation (Matsumiya, et al., 1958a; Takashima, et al., 1958b).

In conclusion, it can be said that the effect of hemoglobin concentration on the hemoglobin-oxygen equilibria indicates that aggregation changes occur in the course of the reaction. From the results of modeling of the oxygenation process, it can be concluded that the oxygenation process in whole blood is not diffusion controlled, but that the controlling mechanism is the reaction kinetics.

Based on the experimental results, a mechanism for oxygenation of whole blood with intact red cells is suggested as follows. Oxygen enters the system by being physically dissolved in the plasma. However, an oxygen gradient from the plasma to the cell exists such that most of the oxygen is drawn out of the plasma into the red cell. Inside the red cell, the oxygen concentration stays at a relatively constant level for much of the oxygenation process. The oxygen entering the cells reacts with hemoglobin to form oxyhemoglobin.

As the hemoglobin becomes saturated, the forward reaction rate is increased and the reverse reaction rate is decreased due to the heme-heme interaction. There is also the possibility of facilitated oxygen transport due to the hemoglobin diffusion as the oxyhemoglobin concentration

gradient increases (Moll, 1969). Thus, oxygen gradient into the cell would be maintained, and the level of oxygen within the cell would remain relatively constant until a sufficiently large amount of hemoglobin had been oxygenated to cause the diffusion terms to become significant. Once this point is reached the oxygen uptake by the cells begins to slow down, and the level of oxygen is allowed to build up in the plasma.

The reactions are all allostearic and result from intramolecular forces acting as intermediate equilibria occur. This reversible oxygenation and deoxygenation of the hemoglobin molecule is extremely rapid, usually requiring less than .01 seconds (Aanong, 1971). Carbon monoxide is also taken up by hemoglobin almost as fast as O_2 but is released at a much slower rate and the half life of the CO in vivo is up to four hours (Bartlett, 1968).

Hemoglobin has four binding sites to which ligands such as oxygen or carbon monoxide can be attached. The reaction velocities for the successive attachments (or dissociations) are not the same, which leads to an S-shaped dissociation curve as mentioned earlier. The dissociation constant for CO is markedly less than that of O_2, with the result that hemoglobin has affinity for CO about 210 times as great as that for O_2. The affinity constant varies with temperature and pH. This is expressed in the well-known Halden equation,

$$\frac{COHb}{O_2Hb} = M \frac{P_{CO}}{P_{O_2}}$$

in which M is the affinity constant. Occupation of one of the four binding sites of hemoglobin by CO makes the molecule hold more tightly to the oxy-

gen it carries, it will release O_2 only when there is a very low partial pressure of O_2. This is expressed as a shift to the left of the dissociation curve. Thus, not only is the total oxygen capacity of the blood diminished by CO, but even that oxygen which is carried is rendered less available to the tissues.

The shift of the dissociation curve of oxyhemoglobin is proportional to the amount of carbon monoxide which is present, and that when COHb is below 10 per cent, the effect upon delivery of oxygen to the tissues would not be very pronounced. Although the carbon monoxide-induced interference with delivery of oxygen to the tissues is of great significance in cases of acute poisoning, it remains to be shown that it is important in the context of community air pollution.

The report takes note of this, mentioning that in vitro studies of the influence of carbon monoxide on oxygen delivery rates of human blood could help to elucidate this matter. The reports rest mainly on the studies of the concentrations of CO in air, the concentration of O_2, the duration of exposure, and the physical activities of subjects (Forbes, et al., 1945).

These studies were able to show quite regular relationships which permitted the development of a nomogram from which COHb values can be predicted from ambient CO concentrations, duration of exposure, and pulmonary ventilation rate or level of physical activity. They also show that the absorption of carbon monoxide is inhibited by increasing the partial pressure of oxygen in the inspired air.

The M value for a species remains relatively constant, depends upon the physiological conditions (Rodkey, et al., 1969). Because the tissue partial pressure of O_2 (P_{O_2}) approximately 40 mm Hg in mixed venous

blood) occurs at a steep portion of the sigmoid curve, under normal physiological conditions oxygen is rapidly dissociated from hemoglobin resulting in a tissue saturation which is maintained with a large oxyhemoglobin reserve near the lower end of the curve. This reserve is the result of the large value of K_4, which permits dissociation only at the upper portion of the curve under normal physiological conditions (Dinman, 1968).

The difference in the partial pressure of oxygen (P_{O_2}) between freshly oxygenated arterial blood P_{O_2} = 100 mm Hg) and mixed venous blood (P_{O_2} = 40 mm Hg) represents a release to the tissues of approximately 5 milliliters O_2/100ml of blood (Bartlett, 1968). The dissociation curve shifts to the right, allowing for a more efficient dissociation of oxygen tension (hypoxic hypoxia) (Lilienthal, 1950). The leftward shift during CO exposure occurs because of the much greater affinity of CO for hemoglobin and in spite of the fact that the amount of oxygen in physical solution in the blood remains close to normal (Bartlett, 1968). The oxygen content of the blood is not only lowered during exposure to CO, but the shift of the oxyhemoglobin dissociation curve to the left decreases the amount of remaining oxygen that is made available to the tissues. Both mechanisms serve to effectively lower the tissue P_{O_2} and hence can create a generalized tissue hypoxia (Dinman, 1968). Some investigators in the field suggested that the great affinity of hemoglobin for CO was a definite survival advantage since this mechanism permits the expedient removal of endogenously-produced CO resulting from the heme catabolism. Some investigators believe that if any acclimatization occurs it is the only result of the accumulated effects of repeated episodes and not merely

a continuous insult by low levels of CO (Rossiter, 1942a; Zoru, et al., 1960b).

Since complete acclimatization to ambient concentrations of CO may not be possible, biological alterations occuring during exposure to CO become suspect as indicators of possible deleterious effects. The same would still be true should acclimatization to CO occur. This reasoning has even led some investigators to believe that death resulting from acute CO intoxication may not be caused solely by simple asphyxia (Suzuki, 1969a; Lilienthal, 1946b) but also by some toxic effect exerted by CO.

Carbon monoxide is eliminated from the body via the lungs. The reaction is a reversible one, and the rate of elimination is governed by the same factors that affect its absorption (Stewart, et al., 1960). The reversible reactions of hemoglobin and myoglobin (Mb) take place in the following reactions:

$$1)\quad CO + Hb \rightleftharpoons HbCO$$

$$2)\quad CO + Mb \rightleftharpoons MbCO$$

The reaction of CO with Mb is physiologically and physiochemically significant, but less important than CO combining with Hb.

The treatment of CO poisoning as described by Smith and Sharp in 1960 was administration of oxygen at high atmospheric pressure (Bour, et al., 1967). The results showed that the period of hypoxia was terminated as soon as the amount of oxygen dissolved in the plasma was sufficient to adequately oxygenate the tissues. The elimination of CO took place more rapidly, resulting in increased alveolar oxygen tension. The unconscious patient, when his breathing is failing or is temporarily arrested, requires

artificial respiration and the method of choice is that of Schater or Holger-Nielsen, admirably described and depicted by Garland (1955). The latter gives a greater tidal volume (580 cm^3) than the Schater back-pressure method (185 cm^3).

Although breathing fresh air may be sufficient, clearance of carbon monoxide is hastened if the patient can breath oxygen mixed with 5 percent carbon dioxide. Haldane (1895) experienced prompt relief when he breathed oxygen along at the conclusion of his experiment. In 1930, following Henderson and Haggard (1927),he recommended the addition of carbon dioxide in a proportion of 7 percent.

Among the most subtle of known physiologic alterations are those which involve shifts in compartmental concentrations of trace metals. New techniques (atomic absorption and anodic stripping voltametry) now allow for the detection of very minute amounts of these important entities, and changes in their departmental concentrations during the course of various disease states, including chronic CO exposure, have been documented (Mazaleski, et al., 1970a; Pecora, 1964b).

Difficulties involved in determining the significance of findings and in the extrapolation of findings to the whole organism have, so far, somewhat undermined the potential values of these techniques. However, the fact that most of the eight essential trace metals participate, physiologically, either as co-factors or activators of enzymes underscores the importance of determining their aberrations in vivo. In fact, any condition precipitating changes in enzyme activity or concentration merits very close scrutiny, and the activity of several enzymes has been found to be altered during CO exposure (Halperin, et al., 1959a; Rozena, et al.,

1959b).

It should be emphasized that these early and many other later investigators have stressed the fact that the effect of CO on man is enhanced by many environmental factors. These have included rate of exercise, high environmental temperatures, altitudes above 2000 feet, and simultaneous exposure to narcotic solvents.

From the viewpoint of health, limiting occupational exposure to CO to a TWA (time weighted average) concentration which will produce no greater than 5 percent COHb may not provide a margin of safety for the employee with clinical symptoms of coronary heart disease. The American Conference of Governmental Industrial Hygienists (ACGIH) Committee on Threshold Limit Values recommended 100 ppm for CO in 1945, and in 1965 the conference recommended that the limit be reduced to 50 ppm, a value that was officially adopted by ACGIH in 1967.

In recommending the lower value the Committee stated that conditions of heavy labor, high temperatures of work 5000-8000 feet above sea level, the threshold limit value should be reduced to 25 ppm. No further benefits under any circumstances could be expected by reducing the level below 5 to 10 ppm since at this concentration one is practically in equilibrium with the normal blood level of one percent COHb. The recommended TLV for CO of 50 ppm is thus based on air concentration that should not result in blood CO levels above 10 percent, which is a development of the sign of borderline effects.

Furthermore, the U.S. Navy established a limit for the average concentration of a continous exposure to CO for prolonged submarine voyage at 25 ppm, and the National Aeronautics and Space Administration

considered 15 ppm CO to be maximum average concentration for space flights.

The American Industrial Hygiene Association has recommended a community air quality guide for CO exposure of 20 ppm as an eight-hour average, which is stated to be equivalent to 3 percent COHb. In addition a limit of 70 ppm has been recommended for a one-hour period, which is also stated to be equivalent to 3 percent COHb.

The recommended limits are based on levels of CO concentration which will not exceed one-half of the 5 to 6 percent COHb prior to tobacco consumption. It was considered that the recommended CO concentrations would permit susceptibles with heart disease to obtain a time based margin of safety prior to reacting the 5 to 10 percent COHb range.

Although the conditions by which a level of 5 percent COHb is reached is variable in individuals, dependent upon such parameters as activity, altitude, length of exposure, and CO concentration, as well as individual differences in the CO uptake, the smoker who engaged in sedentary activity will approach this level in eight hours if continuously exposed to 35 ppm of CO.

Basis for Recommended Standard

The recommended standard is based on the cardiovascular and behavioral evidences which generally documents the initiation or enhancement of myocardial alterations in individuals with coronary heart disease (CHD) who exposed to CO concentrations sufficient enough to produce a COHb level greater than 5 percent. Recent extensive studies are demonstrating restricted coronary blood flow and myocardial lactate production under such circumstances and concerning CO exposure and exercise

of patients with angina pectoris are germane to the recommended standard (Knelson 1972a; Ayres, et al., 1969b).

The synergistic relationship imposed by chronic cigarette smoking and concomitant exposure to CO upon the enhancement of such detrimental myocardial alterations were reported by the Surgeon General (The Health Consequence of Smoking, 1971). Based on the available evidence, the imposition of a COHb level of 5 percent on an active worker with clinical or asymptomatic CHD is unwarranted.

There are certain difficulties in measuring and extrapolating 5 percent COHb of the worker in relation to a meaningful ambient CO concentration in the work place. At first, air sampling methodology must depend on the statistical techniques to develop an eight-hour, time-weighted, integrated analysis. Secondly, the rate of the activity of the worker will increase the exposure to CO by decreasing the length of time to COHb equilibrium and by maintaining a higher COHb level in the active worker than in the sedentary worker who has not reached equilibrium. The significance of the first difficulty can be practically resolved by an air sampling protocol which will insure that sufficient quantities of such samples are taken to provide a reliable, statistical estimation of proposed eight-hour standard. The activity factor can be minimized by an equation which takes into consideration, among other parameters, the activity of the worker in terms of alveolar ventilation rate and pulmonary diffusion rate (Coburn, et al., 1965).

The recommended TWA standard of 35 ppm CO is based on a COHb level of 5 percent, which is the amount of COHb that an employee engaged in sedentary activity would be expected to approach in eight hours during

continuous exposure. The ceiling concentration of 200 ppm is based on the restriction of employee exposure to CO to transient excursions above 35 ppm which would not be expected to significantly alter his level of COHb.

The recommended standard is based on the utilization of the CO for an eight-hour work day. The applicability of the equation for this purpose has been validated by a study of Peterson and Stewart (1970).

While the values assigned to several of variables in the equation by the investigators were for sedentary individuals (e.g., alveolar ventilation rate of 6 liters/minute, CO pulmonary diffusion rate of 30 milliliters/minute/mmHg), it is recognized that the range of activities of the worker in the work place may vary considerably. It is incumbent on the employer to recognize the effect that the level of activity has on the uptake of CO and judiciously evaluate the exposure of his employees and limit their activities accordingly. In addition, the employer must give special consideration to limiting the activity of employees exposed to CO at high altitudes in order to compensate for dual loss in oxygen-carrying capacity of the blood.

The Environmental Protection Agency (EPA), under provisions of the Clean Air Act (PL 91-604), promulgated national primary and secondary air quality standards on April 30, 1971. The primary and secondary standards for CO are:

1) 10 milligrams per cubic meter (9 ppm) - maximum eight-hour concentration not to be exceeded more than once per year.
2) 40 milligrams per cubic meter (35 ppm) - maximum one-hour concentration not to be exceeded more than once per year.

Promulgating this standard the Administrator of EPA made the following statement (Stewart, et al., 1970) concerning comments raised about the evidence used to support the proposed standard:

> In the comments, serious questions were raised about the soundness of this evidence. Extensive consideration was given to this matter. The conclusions reached were that the evidence regarding impaired time-interval discrimination had not been refuted and that a less restrictive national standard for CO would therefore not provide the margin of safety which may be needed to protect the health of persons especially sensitive to the effects of elevated carboxyhemoglobin levels. The only change made in the national standards for CO was a modification of the 1-hour value. The revised standard affords protection from the same low levels of blood carboxyhemoglobin as a result of short-term exposure. The national standards for carbon monoxide, as set forth below, are intended to protect against the occurrence of carboxyhemoglobin levels above 2 percent. It is the Administrator's judgment that attainment of the national standards for carbon monoxide will provide an adequate safety margin for protection of public health and will protect against known and anticipated adverse effects on public welfare.

The air quality standard is designed to protect the population-at-large and takes into consideration 24-hour per day exposure of the young, the old and the ill.

Fibrinolysis and Thrombosis

The concept of thrombosis was developed largely through anatomic observations. For centuries, the development of the concept was hindered by the lack of general recognition that blood in the heart and blood vessels may solidify after death as well as during life (Millikan, 1958a; U.S. Department of Health, Education and Welfare, Public Health Service, 1962b). The confusion that existed until well into the nineteenth century is best illustrated by the well known history of the problem of heart polyps. The problem concerned the distinction between polyps that were true

thrombi and possessed "vitality" and polyps that were simply postmortem coagula (Eisenberg, et al., 1964). The pathologic nature of a thrombus was determined mainly by these anatomic observations: evidence of degeneration during life, lamination, and adherence to the vessel wall (Bronte, et al., 1964a; Eisenberg, et al., 1964b; Hodge, et al., 1966c). The recognition of the effects of ischemia caused by thromboembolization made it quite clear that thrombi have vitality (Wylie, 1962).

Experimental studies established that thrombi of a distinctive structure from inflowing blood from constituents of blood (Dorn, 1959a; Kallner, 1958b). Natural thrombi were found to have the same basic structure and constituents, indicating a similar mode of formation (Kallner, 1958a; Katsuki, et al., 1964b). Variations in structure, although observed and beautifully illustrated as early as 1805 by Jones were not fully understood until it was shown how disturbances of blood flow influence the structure and development of a thrombus (Marshall, 1964a; Aurell, et al., 1964b; Ueda, 1962c). Welch, in his noted review of 1899, summarized the experimental and morphologic studies that led to the concept of thrombosis as we know it today (Eisenberg, et al., 1964). His definition of a thrombus, "a solid mass or plug formed in the living heart or vessels from constituents of blood" is widely used (Takahashi, et al., 1961). Although the main aspects of the nature of a thrombus were worked out by the end of the nineteenth century, there have been contributions since then, especially by the application of recently developed techniques such as electron microscopy, enzyme histochemistry and immunofluorescent microscopy.

Formation of fibrin is an important stage in hemostasis, thrombosis and tissue repair because the fibrin formed solidifies the hemostatic

platelet plugsand provided a matrix for the formation of reparative connective tissue with fibroblastic proliferation and growth of capillaries. Ultimately, the fibrin has to be removed, mainly by the process of fibrinolysis, in order to restore normal conditions. Fibrinolysis is caused by the blood proteinase, plasmin. Plasmin (fibrinolysin) is formed from a precursor, plasminogen (profibrinolysin), abundantly present in blood plasma (Kaulla, 1963). Plasminogen is transformed to plasmin by a variety of activators. Inhibitors regulate the activation of plasminogen as well as the effect of plasmin (Fearnley, 1965a; Konttinen, 1968b). In the living organism, a dynamic equilibrium, or hemostatic balance, exists between fibrin formation and resolution. Disturbances in this balance may lead to impaired or to excessive fibrin formation and, consequently, to impaired hemostasis or pathological tissue repair (Sherry, 1969). The involvement of the mechanisms of blood coagulation and fibrinolysis in the regulation of tissue repair signifies them as fundamental physiological processes, important not only in hemorrhagic disorders and in thromboembolic diseases but playing a role in a multitude of pathological conditions (Frohlich, 1972). Fibrinolysis bears an obvious relation to thrombosis: the end product of thrombosis, the thrombus, depends on fibrin for its tensile strength and structural integrity (Sherry, 1969). However, the relationship between the two processes is considerably more intimate, in that it is evident that a thrombus is not static, but rather provides an interface at which the dynamic events of thrombosis and fibrinolysis continuously overlap (Astrup, 1956). As a consequence, it is increasingly apparent that the study of thrombosis must include fibrinolysis as an integral part.

Rapid strides were made in understanding how the fibrinolytic enzyme

system worked when it became apparent that the fibrinolytic activity of plasma was directly related to its plasminogen activator content (in fact, was measure of it), (Albrechtsen, 1956) that plasminogen was deposited, and (Ambrus, et al., 1962) that plasminogen activation in the immediate proximity of fibrin (e.g., in the interstice of a clot) was the most sensitive mechanism for fibrinolysis (Kwaan, et al., 1965). This has led to the development of a hypothesis (Kirk, 1962a; Müllertz, et al., 1954b; Todd, 1960c; Sherry, 1969d) that appears to account for the facts: in vivo, plasminogen exists as a two-phase system, a soluble phase in the body fluids (plasma plasminogen) and a gel phase in thrombi and fibrinous deposits (Glas, et al., 1967a; Janda, 1965b; Coccheri, et al., 1961c). The effect of activators in the two phases and the consequences of plasminogen activation in the two sites are dissimilar (Sherry, 1969). Minor or slow activation of plasma plasminogen, because of the presence of inhibitors, will not result in detectable signs of plasma proteolysis, because the enzyme is effectively inhibited on its formation; but rapid activation of plasma plasminogen produces excessive plasma proteolysis, resulting in the rapid degradation of fibrinogen, the most abundant available substrate. However, activation of gel phase or clot plasminogen, resulting from diffusion or incorporation of the activator into the clot, produces fibrinolysis, (Ibid.) for here the enzyme is activated in the presence of fibrin, which is the only substrate available, and the reaction appears, at least initially, to be independent of inhibitors in the body fluids.

These considerations attach great specificity to fibrinolysis, in that, under physiologic circumstances, fibrinolytic phenomena appear to be regulated by the concentration of plasminogen activator. Plasminogen

activator is present in blood, and various body fluids and secretions such as urine, milk, saliva, tears, seminal fluid and cervical mucous.

Our present knowledge of the various activating and inhibiting steps within the fibrinolytic enzyme system is represented in the following schematic outline. Following an appropriate stimulus, activator

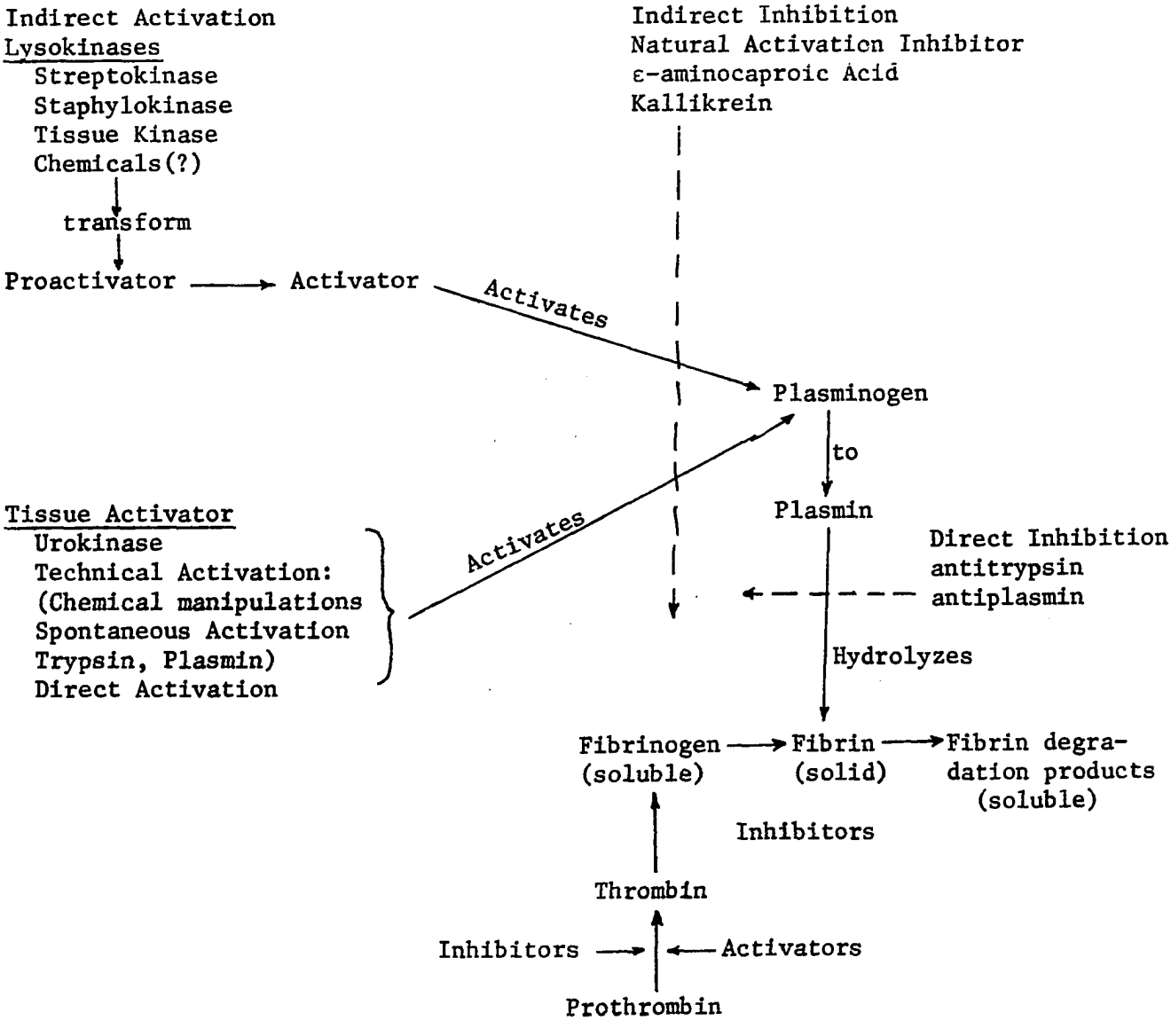


FIGURE 1. Schematic Pathway of Fibrinolysis

is released transiently into the circulation and directly raises the clot-dissolving activity of the plasma by its ability to activate gel phase plasminogen, but without invoking the consequences of increased plasma proteolysis (Ashford, et al., 1967). The mechanism is particularly effective if significant quantities of activator are present when fibrin formation occurs, (Sherry, 1969) under these circumstances, the activator is incorporated into the clot while it is forming, and the subsequent widespread activation of clot plasminogen leads to very rapid fibrinolysis.

The fibrinolytic mechanism appears to be continuously active, and it is dynamic in response to stimuli. Enhanced fibrinolytic activity is observed frequently in some diseases--e.g., hematologic malignancies, cirrhosis of liver, and various infections. The physiologic and pharmacologic stimuli also producing changes in fibrinolytic activities such as electroshock, pneumoencephalography, hypoglycemia, ischemia, anoxia, intense exercise, and parenteral injections of epinephrine, acetylcholine, nicotinic acid, or pyrogen (Duckert, et al., 1960). Fibrinolysis is viewed as a local phenomenon for years but there is enough evidence to believe that plasminogen activator is released into the circulation at the site of ischemia or other acute vascular change, by vasodilatation or vasoconstriction (Fearnley, 1965). Plasminogen activators (Kaulla, 1963), are derived from both exogenous and endogenous sources. Most exogenous activators consist of bacterial products such as streptokinase and staphylokinase. The endogeneous or physiologic activators, are found in blood and tissues. The tissue activators are found in all organs of the human body, with the exception of the liver. It was recently

observed that in the kidney, the tissue activator is associated with lysosome traction (Ali, et al., 1965). Further information on the chemical nature of the physiologic blood and tissue activator is very limited, except in the case of urokinase, an activator present in urine (Walton, 1967). The identity or non-identity of urokinase with tissue and blood plasma activator is being discussed.

The inhibitors of the fibrinolytic enzyme may also be of exogenous or physiologic origin. There are several synthetic inhibitors. Epsilon amino-caproic acid is the most significant one (Norman, 1966). It possesses both antiactivator and antiplasmin qualities, and which is known to be an effective anti-fibrinolytic agent in the treatment of hemorrhage due to pathologically increased fibrinolytic activity. Another exogenous inhibitor which has proven value in clinical application is Trasylol^{R}, and a small peptide extracted from bovin salivary glands and lung tissue. There are two antiplasmins that are referred to as alpha-1 and alpha-2 antiplasmin (Ibid.).

Alpha-1 antiplasmin possesses a slow and non-dissociable inhibiting effect. It is temperature dependent, and is destroyed after being incubated at 56^{o}C for 30 minutes, dissociable from plasmin and much less temperature sensitive. Recently the role of alpha-2 macroglobulin has been emphasized. Antiplasmin activity can be demonstrated in various other tissues, such as lung, liver, and even within seminal fluid, ovarian follicles and cervical mucous (Kaulla, 1963).

Fibrinolysis has been inhibited in man for therapeutic purposes, both for systemic states of excessive fibrinolysis and to control local fibrinolytic agents, epsilon-aminocaproic acid, trasylol, p-aminomethylcyclo-

hexanecarboxylic acid, (Cutler, 1967), and p-aminomethylbenzoic acid (Markwardt, 1965).

Physiologically, fibrinolysis is accomplished without a significant increase in plasma proteolysis. But sometimes there is excessive digestion of fibrin or fibrinogen. This state is associated with an acute or chronic coagulation disorder, and when the onset is sudden, a serious hemorrhagic diathesis may develop rapidly with severe bleeding. Although the blood of man or animal suffering from this disorder frequently demonstrates multiple coagulation defects, among the most common ones poor and slow clotting, even after the addition of thrombin; and, if a clot forms, it is loose and friable. The clot may undergo spontaneous dissolution in a matter of minutes or hours, and that makes the syndrome recognizable; it is referred to as "pathologic fibrinolysis" or "fibrinolytic bleeding." The severity of this disorder can be readily attributed to the particularly ineffective form of hemostasis present, clotting is slow and inadequate, and the clot may later dissolve (Sherry, 1969).

The understanding of this hemorrhagic diathesis has come from studies that demonstrated that the products of the proteolytic digestion of fibrinogen and fibrin interfere with blood clotting, and that the addition of such proteolysis products to normal blood, in vitro the abnormal blood clotting seen in the fibrinolytic disorders (Kuller, et al., 1967; Triantaphyllopoulos, et al., 1965b). The appearance in plasma of breakdown products during states of excessive fibrinogen or fibrin proteolysis can be demonstrated most readily by immunologic techniques (Merskey, et al., 1966) and the fragments can be characterized as the type by immuno-

electrophoresis (Marder, et al., 1969). However, under clinical conditions, the coagulation disorder can be screened for most simply by measuring the thrombin clotting time. In the presence of excess thrombin, the thrombin clotting time is virtually a measure of the polymerization time, as a result, the thrombin clotting time, although lacking specificity, is useful. Thus, the immediate problem in the fibrinolytic disorders is related not to the level of fibrinolytic activity or plasmin in the circulation but to the presence of abnormal amounts of fibrinogen or fibrin breakdown products, which arise as a result of the excessive digestion of fibrinogen or fibrin. These breakdown products, in high concentrations seriously interfere with the normal clotting mechanism and precipitate a severe hemorrhagic diathesis. The fibrinolytic enzyme measurements consist mainly of indirect determinations. Overall blood fibrinolytic activity is simply observed following the incubation at $37^{o}C$ of the diluted or undiluted whole blood or plasma clots. The decreased fibrinolytic inhibitor activity after dilution causes the faster dissolution of blood clots.

There are several ways to measure plasminogen activators. One method is by observing lysis time of the clotted euglobulin fraction which contains most of the activator present in the blood. Euglobulin fraction is obtained by lowering plasma ionic strength and pH of plasma. The euglobulin precipitate is recovered practically free of antiplasmin. The euglobulin lysis time is inversely related to the activity of plasminogen activator. Activation of plasminogen to plasmin results in the digestion of the fibrin network of the euglobulin clot and eventually lysis of the clot without any interference by antiplasmin. In the second method, the

excess quantity of plasminogen of plasminogen-rich fibrin clot serves as a substrate for testing plasminogen activator. The amount of plasminogen contamination found in a solution to be tested for activator activity would be a negligible when compared to the plasminogen already present within the substrate. Consequently the extent of fibrin digestion will reflect the activator activity present in the activation of plasminogen to plasmin (the latter causing the dissolution of the fibrin substrate). The plasminogen-fibrin substrate is clotted in petri-dishes upon which the test solution is applied. The resulting lysis zones are a measure of activator activity. The method is known generally as the fibrin plate technique. In the third method, the moderately reduced fibrinogen level measured by the methods of Partenjez or Kjeldahl. In the fourth method, excessive amount of immunologically identifiable fibrinogen breakdown products measured.

The plasminogen determination is also indirect. An excess of purified plasminogen activator such as urokinase or streptokinase is used to activate plasminogen to plasmin. Plasmin in turn is allowed to digest a protein substrate, casein, used in most cases. The quantity of protein substrate digested by formed plasmin will be measure of plasminogen.

Plasmin can be measured directly. The substrate used can be either fibrin or other proteins such as casein or synthetic amino acids esters. Antiplasmin is indirectly determined by measuring the remaining plasmin activity after standard period of incubation with test solution having a known concentration of plasmin. Anti-plasmin activity is the measurement of the ability to neutralize plasmin.

Control of the primary fibrinolytic disorders accomplished by administrating the synthetic amino acid, epsilon-aminocaproic acid. Shortly after the oral administration of this agent in appropriate dosage (loading dose of 100-200 mgm/Kgm/day) plasma plasminogen activation ceased and fibrinolytic activities decreased spontaneously (frequently dramatically).

The secondary fibrinolytic disorders are seen in association with the disseminated intravascular coagulation syndrome (also referred to as "diffuse intravascular clotting," "acute defibrination syndrome" and "consumption coagulopathy"). Present evidence indicates that most of the acutely acquired hypofibrinogenemic disorders encountered clinically (i.e., those in which plasma fibrinogen is strikingly reduced) are due to disseminated intravascular coagulation. Fibrinolysis contributes significantly to this disorder, usually as a secondary local response but sometimes because of simultaneous release of tissue thromboplastin and tissue activator into the circulation (Kowalski, et al., 1965).

Because of the overlapping findings and the lack of a specific diagnostic test, the differentiation between protracted intravascular clotting complicated by secondary fibrinolysis (fibrin degradation) and primary fibrinolytic disorder (fibrinogen degradation) may be extremely difficult. Nevertheless, recognition of the problem should lead to the development of new laboratory methods capable of making this distinction accurately, so as to provide a more scientific and effective therapeutic approach.

At present, reliance on good clinical judgment and careful laboratory study (of the amount of increased circulating fibrinolytic activity, the severity and nature of the coagulation deficiencies, decrease in

platelet numbers, etc.) will help to resolve this difficulty.

At the present time, our knowledge of biological chemistry is far advanced in results of fibrinolytic enzyme system research. The molecular basis and kinetic character of several fibrinolytic enzymes (plasminogen, plasmin and urokinase) have been extensively investigated. This information, unfortunately, is in some sort of comparative imbalance with that available on the physico-chemical significance of the fibrinolysis system. The role of the fibrinolytic system in body physiology is not yet well defined. Fibrinolysis as the physiological counterbalance to coagulation is being discussed, but not yet either proved or disproved. This role of fibrinolytic activity as a counterbalance still remains as a hypothetical statement with little additional information forthcoming from the last two decades. Knowledge about the site of origin of fibrinolytic enzymes and the regulating mechanism of this system is also very sketchy.

Intravascular clots are one of the most frequent causes of death (Kaulla, 1963a; Merskey, et al., 1963b). Theoretically, the formation of intravascular clots should be counteracted by increasing the body's fibrinolytic potential. A significant future contribution to preventative medicine would be the discovery of an ideal therapeutic agent for preventing this cause of death.

It became apparent in the last decade that a considerable body of pertinent information was being obtained which served to tie together the relationship between interfacial phenomena, thrombosis and hemostasis. Available information in this area had been acquired by a very small group of investigators who exchanged and complemented each other's findings,

but did so in a very narrow spectrum without wide dissemination of their results. At present, considerable effort and interest were made by the members of such diverse specialties as vascular surgery, hematology, biochemistry, biophysics, and engineering.

If fibrinolysis counter-balances blood coagulation, intravascular clotting should represent an imbalanced stage. Production of more information along these lines will better the chances of solving this problem.

The Present Knowledge of the Roles of Vascular System and Circulating Blood in Relation to the Fibrinolytic Enzyme System

According to Mole, the fluidity of blood after sudden death already had been recognized in 1871 by Virchow (Loewy, et al., 1954). In 1906 Morawitz showed that blood obtained at postmortem was fluid, free from fibrinogen, and at times fibrinolytic. Fidon, Gautier and Martin in 1908 and Oki in 1934 demonstrated the occurrence of fibrinolysis in experimentally asphyxiated dogs. Yudin in 1936 took the advantage of the unclotted blood in persons succumbing by violent death for transfusion purposes. Mole made a further excellent study on postmortem blood (Mole, 1948). He found in those who died from infection and cachexia, that the blood was clotted, whereas, in the majority of sudden deaths of previously healthy persons, the blood was fluid. Absence of fibrinogen and presence of fibrinolytic activity in these blood samples was confirmed. The lytic activity was centripetally decreased, blood from limbs being more active than blood from the heart. Portal blood behaved similarly to heart blood and there was no difference between blood from the right or left

heart chambers. No evidence for the release of fibrinolytic activity from any particular organ was obtained. There was an approximate inverse relationship between the activity of fibrinolysis and the diameter of the vessel from which the blood sample was obtained. Mole suggested that fibrinolysis might be the product of the vascular endothelium. He also mentioned leucocytes as another possible source of fibrinolytic activity. Tagnon and his associates studied 22 patients with peripheral vascular collapse (rapid pulse, low blood pressure, cold and sweaty extremities) due to various causes. Eight patients had increased fibrinolysis (Tagnon, et al., 1946). Of these eight patients, four were in severe hemorrhagic shock, three were extensively burned and one probably was poisoned by a barbiturate. Then these authors induced hemorrhagic shock in 13 anesthetized dogs. Six dogs died instantly from improper handling, five of the remaining dogs showed a marked increase in fibrinolysis two hours after the onset of shock. In another group of their experiments, five dogs were bled to death within five to eighteen minute periods. Blood samples were withdrawn every one to two minutes. Fibrinolysis was observed to develop in three dogs. It was concluded that shock could induce fibrinolysis in certain cases while in the rest, the time of sampling might have been inappropriate. The fibrinolytic enzyme could originate either from within the plasma or be derived from cellular elements by damage to the tissue. Kwaan and McFadzean were the first to induce local fibrinolysis within the body (Kwaan, et al., 1956). They showed increased fibrinolytic activity in blood withdrawn from an ishcemic arm compared to the sample obtained from the opposite control arm. This fibrinolysis was increased progressively

in the intensity in a centrifugal manner as shown in successive samples withdrawn from the same ischemic arm. Investigators suggested the possibility of release of fibrinolytic activity from small arterial trees or from the capillary beds. Paravenous or intravenous injection of adrenaline or acetylcholine under conditions preventing rapid absorption of the drugs (by pre-application of tourniquet at diastolic pressure above the injection area) induced fibrinolytic activity in that vein. This induction could not be observed when the arterial flow was obstructed. Atropine also prevented this induction. Thrombi produced in an isolated segment of the rabbit's ear vein were spontaneously lysed. Therefore, it was suggested that fibrinolytic activity is released from the venous wall and that direct stimulation of cholinergic receptors were involved in this release. Pandolti and his associates, on the other hand, were able to show increased blood fibrinolytic activity after venous stasis at a pressure mid-way between systolic and diastolic pressure. Lysis of thrombi in opened segments of rabbit ear veins was enhanced by injection of adrenaline and serotonin (5-hydroxy-tryptamine), whereas atropine delayed it (Kwaan, et al., 1958a; Warren, 1963b). The enhancement by serotonin was more pronounced if given by the paravenous route rather than intravenously. Corticotrophin inhibited the effect of the serotonin but not of adrenaline. Corticotrophin is claimed to have an inhibitory effect upon monoamine oxidase, an enzyme which is able to oxidize serotonin. This explains the better stimulating effect of serotonin when given paravenously. Atropin exerted a vaso-dilating effect while adrenaline and serotonin caused vasoconstriction. A hypothetic statement was made that vasoconstriction, especially of the vasa vasorum, might cause ischemia

of the venous wall and thereby trigger the release of plasminogen activator. This, however, is at variance to what the authors had suggested in their previous report concerning the requirement of flow through the vasa vasorum for releasing plasminogen activator (Kwaan, et al., 1957). The nature of the fibrinolytic enzyme system involved was not known, but the authors suggested the possibility of it being a plasminogen activator.

Todd established the fibrin slide technique to detect fibrinolytic activity histologically in tissues by adoption of the fibrin plate method of Astrup and Lassen (Todd, 1958a; Astrup, et al., 1952b; Lassen, 1953c). He further investigated practially all the organs within the body and found that the fibrinolytic activity within the tissues after 30 minutes incubation on fibrin slides was related only to veins and venules, except in the lung where it was related to the pulmonary artery (Lassen, 1953). In arteries the activity was localized only in the vasa vasorum, none in the intima. The desquammous endothelial cell from venous intima also caused lysis areas on the fibrin slide, but the remaining underlying layers of the veins had no activity. By using heated and unheated fibrin plates, Todd also proved that the fibrinolytic activity within the active tissues was due to release of a "plasminogen activator" (Chakrabarti, et al., 1963). Warren was, however, able to demonstrate lytic areas on fibrin plates caused by endothelial strips from the pig ox and rat aortae after three to five hours incubation while those from the inferior vena cavae showed activity after only one to two hours. Plasminogen activator in the arterial wall also was found to be related to the vasa vasorum possibly being derived from their endothelial cells. Kwaan induced clots in the femoral vein of the rat using thrombin, serum and

sodium morrhuate. Thrombin and serum clots lysed after four hours, but sodium morrhuate thrombi did not lyse until recanalization took place with the formation of new active endothelial cells. Sodium morrhuate caused endothelial damage which abolished its fibrinolytic activity. Astrup performed a series of quantitative analysis of the plasminogen activator activity by measuring the activity of potassium thiocyanate extracts of individual tissue layers of the aorta and inferior vena cava in numerous subjects: humans, rats, rabbits, dogs, oxen, horses, pigs and monkeys (Astrup, et al., 1963a; Albrechtsen, et al., 1959b; Coccheri, et al., 1961c). Celander's group measured plasminogen activator released from everted external jugular veins and femoral veins of the dog by assessing the amount of digestion of a plasminogen rich fibrin clot. They found that veins with a large caliber have more activity than smaller veins, and that the fibrinolytic activity was inhibited by epsilon amino caproicacid (Celander, et al., 1966a; Messer, et al., 1962b). Kwaan, in his report on fibrinolytic activity and its correlation to tissue repair, states that demonstrable activity was found to be related to the newly formed capillaries of the repairing granulation tissues (Kwaan, 1966). His investigations included: implanted homologous plasma clot in rats, active ulcerative colitis, human myocardial infarction and finally experimental liver cirrhosis in rats. Especially with the implanted plasma clot, a serial study was continued from two days to two months after implantation. In the initial state (within four days of implantation) when polymorphonuclear leukocytes and other acute inflammatory cells began to infiltrate, fibrinolytic activity and weak proteolytic activity were demonstrated. After one week, further increase of plasminogen activator was

found in capillary buds sprouting from the clot. After two weeks the implanted clot had disappeared but high plasminogen activator then decrease progressively and at four to six weeks the fibrinolytic activity was no longer found in all capillaries and a longer incubation time was required to induce areas of lysis. In experimentally induced cirrhosis of the rat liver, the fibrinolytic activity was also found to be associated with capillaries in the fibrotic area. Kwaan therefore suggested that the marked fibrinolytic activity is associated with young capillaries and appears to play a role in the process of tissue repair. From these observations and findings by other investigators, it can be concluded that endothelial cells are possibly an important source of plasminogen activator both in blood and tissues.

Barnhardt and Riddle, using a fluorescent antibody technique, demonstrated the presence of plasminogen within eosinophilic granules of bone marrow cells. When the cells became mature the plasminogen content increased. Barnhardt and Riddle suggested the possibility that the eosinophilic cells of the peripheral blood are responsible for the transport of plasminogen, when its needed, to the general circulation and the tissues (Barnhardt, et al., 1963).

Kunzer and Haberhausen demonstrated the presence of plasminogen activator in the stromata of the red blood cell hemolysate, although hemoglobin showed no fibrinolytic activity (Kunzer, et al., 1963). They suggested that erythrocytes might play a role in clot dissolution. That the red cell stroma does indeed contain plasminogen activator was later confirmed by Tyminski and Czestochowska (Ibid.). They also observed the presence of plasminogen and plasmin in this material.

Both fibrinolytic enzymes and fibrinolytic inhibitors were rather definitely demonstrated within the formed elements of the blood. There is still no information available concerning their definite roles in the fibrinolytic system. However, in various blood diseases changes of the blood fibrinolytic activity has been observed. Increased fibrinolytic activity has been reported both in acute and chronic leukemia (Fearnley, 1965).

In the lymphoma group which includes Hodgkin's disease, lymphosarcoma and reticulum cell sarcoma 20 percent of 125 cases studied showed increased fibrinolysis. The plasminogen level in both leukemia and lymphoma was lower than normal, an apparent consequence of increased fibrinolytic activity resulting in a using-up of plasminogen. The observation of increased fibrinolytic activity in both leukemia and patients is very interesting. Unfortunately, all the fibrinolytic determinations were done only in plasma. No data was reported on fibrinolytic enzyme within leukemic and lymphoma cells, with the exception perhaps of Rosenthal who mentioned the absence of fibrinolytic activity in suspension and extracts of the promyelocytic cells from patients with acute promyelocytic leukemia (Rosenthal, 1963).

It is worthwhile to point out that the vascular wall, plasma and formed elements of the circulating blood, are enriched with fibrinolytic enzymes. Whether these represent the sites of storage or of actual synthesis of the fibrinolytic enzymes has not yet been elucidated.

From various observations, it is apparent that both the autonomic nervous system and physiologic reflex mechanisms participate significantly in the plasminogen activator release. Cholinergic vasodilation is probably

one of these neurogenic mechanisms. However, other unexplained mechanisms also seem to participate in activator release, namely electroshock and atropine induced fibrinolysis. This certainly requires further investigation.

The fibrinolytic system has an apparent inter-relationship not to one individual organ but to several different organs. Although the evidence for this deduction was mostly in pathologic conditions, the important conclusion that possibly the regulation mechanism of the fibrinolytic system under hypoxia stands firm and challenging. Abnormal function in many organs (under hypoxia, chronic or acute exposure to CO) seems to induce an imbalance of fibrinolytic enzyme system. Increased fibrinolytic activity under hypoxia was observed in numerous pathological conditions. However, there are still many abnormal physical states involving the function of a multitude of organs under hypoxic conditions where there is no information on their relation to the fibrinolytic system.

Plasminogen and antiplasmin were found to fluctuate insignificantly during changes of fibrinolytic activity. In contrast, the plasminogen activator was clearly demonstrated to be associated with and appeared to be responsible for changes of fibrinolytic activity in both increased and decreased fibrinolytic states. This indicates that the plasminogen activator is probably the most important variable component in the fibrinolytic enzyme system. Generally speaking, the regulation of any metabolic pathway is known to be primarily effected by so-called "key enzymatic steps." The regulation is found to be in their enzymes ("key enzymes") whose activities are variable, thus maintaining that pathway in a balance state (Anderson, 1973). This could also apply for the fibrinolytic system.

Plasminogen activator would fit beautifully into the concept of "key enzyme" as the main regulator of the total fibrinolytic system in the body, although the modulating factors are not yet well-defined. Plasminogen activator is found in practically all organs and tissues. This indicates the significance of the plasminogen activator, although its function in tissues is not yet known. Our hope is to obtain enough information to further the understanding of the basic relationship between hypoxic conditions either chronic or acute and fibrinolytic enzyme system, and in addition, the function of this fibrinolytic enzyme system and relation to the coronary thrombosis.

Finally, it is hoped that the results obtained in the investigations to be reported may make a significant contribution to widening the knowledge and increasing the understanding of the hypoxia and its effects on fibrinolytic enzyme system within the body.

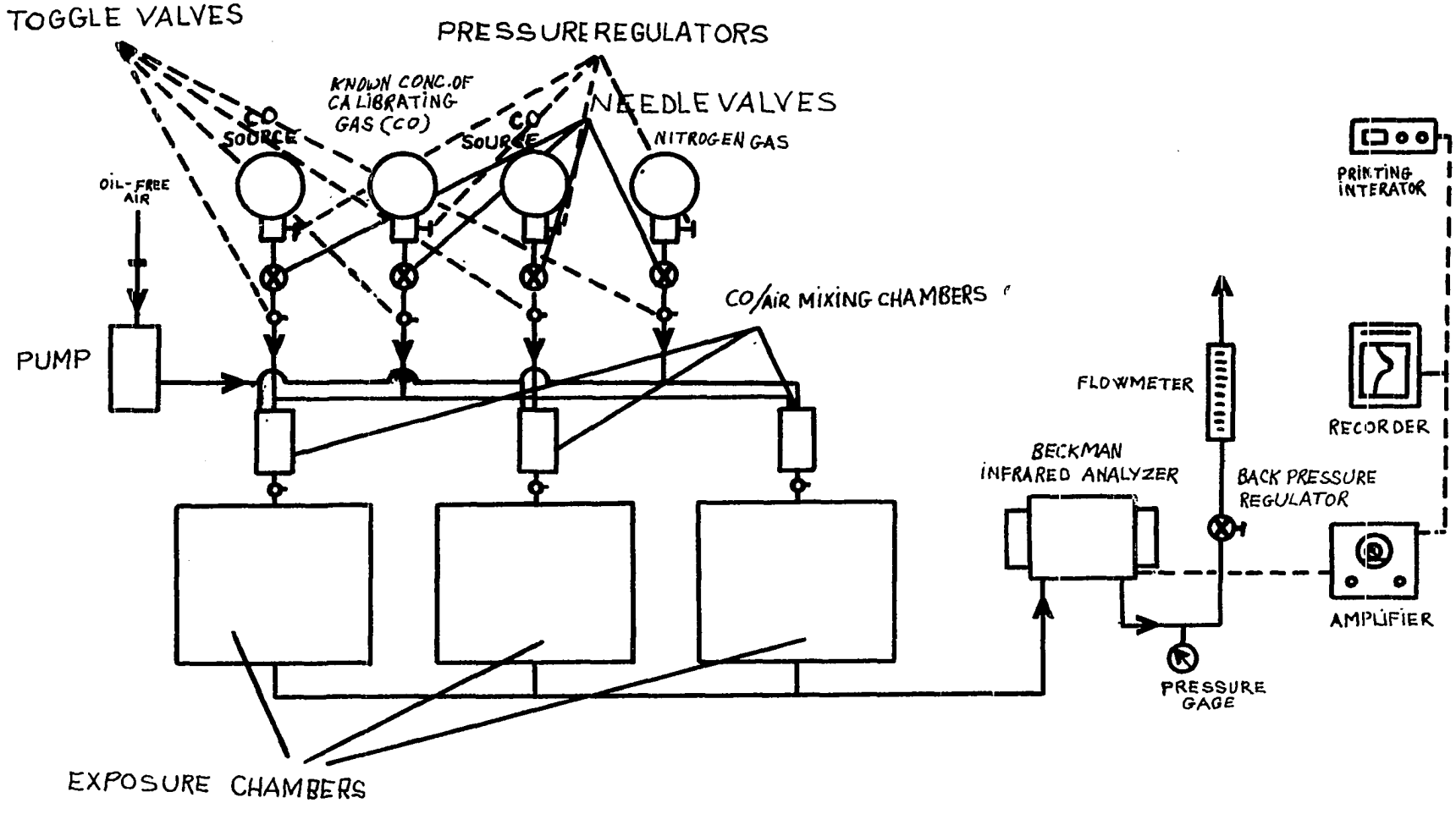
CHAPTER III

EQUIPMENT, METHODS AND PROCEDURE

3.1 Animals: Male New Zealand white rabbits (3.50-4.50 kgm) were maintained on a diet of Purina Laboratory Chow and water. The water was treated with subclinical dose of Purina tylosin tartrate (0.5 gm per gallon) to decrease incidence of respiratory infection commonly associated with laboratory animals.

3.2 Exposure Chambers: Air-tight environmental chambers were used in which rabbit cages could be placed and through which gas mixtures could be passed. One of the chambers was of simple flow-through design which allowed for the maintenance of a normal air environment at one atmosphere. The other chambers were incorporated into a closed environmental system, each having the dimensions of 5 x 3½ x 3 ft. Each chamber was operated at a slight negative pressure and was capable of holding four metabolic rabbit cages without crowding. Oil-free compressed air and CO were premixed in a laboratory fabricated mixing chamber containing small glass beads, and this CO/air mixture was diffused throughout the chambers through small holes in plastic pipe within each chamber. The system was maintained with physical constants: (a) the constant temperature, (b) the constant positive pressure, (c) the constant negative pressure system which brings the effluent gases from the

FIGURE 2. Sketch indicating principles of operation of the exposure chambers, measurement of CO concentrations, calibration and recording system.


TOGGLE VALVES
PRESSURE REGULATORS
NEEDLE VALVES
KNOWN CONC. OF CALIBRATING GAS (CO)
CO SOURCE
CO SOURCE
NITROGEN GAS
OIL-FREE AIR
PUMP
CO/AIR MIXING CHAMBERS
FLOWMETER
BECKMAN INFRARED ANALYZER
BACK PRESSURE REGULATOR
PRINTING INTERATOR
RECORDER
AMPLIFIER
PRESSURE GAGE
EXPOSURE CHAMBERS

animal chamber to the exhaust tunnel, (d) the constant humidity. The tanks were balanced to maintain the 50 ppm CO level for 24 hrs. Carbon monoxide was measured by Beckman Model 315 A(L) infrared gas analyzer (41-inch cell length) constantly (constant concentration of 50 ppm) to each chamber. Commercial grade CO having 98 per cent purity was purchased from the Matheson Scientific Co. and used as the exposure gas. Positive CO pressure was maintained by a single stage regulator.

The CO source tank was connected to a gas mixing chamber by a line from a Mine Safety Appliance single stage regulator on the tanks. The compressed air was connected from the compressed air source to the gas mixing chamber through a separate aperture while another aperture permitted the gas sampling line to exit from the chamber for quantitative analysis. (Figure 2.)

The CO infrared analyzer could be adjusted to monitor the exposure chamber either continuously or, by manual adjustment, intermittently. During the night and early morning hours the exposure chamber was monitored on a continuous basis. Prior to exposure, the analyzer was calibrated with 100 percent nitrogen and then with CO of known concentration. Following calibration of the analyzer, the mixture of CO compressed air within the exposure chamber could be adjusted for proper proportions to meet the desired CO concentration. Once the calibration was completed, the desired CO concentration could be maintained within ±5 ppm using this exposure arrangement. Carboxyhemoglobin and hemoglobin concentrations were measured by utilizing the IL-182 CO-oximeter.

This procedure involved the combination of a sensitive and accurate absorption spectrophotometer with an analog computer. Three precision

interference filters were used as monochromators to measure the blood oxygen, carbon monoxide and hemoglobin concentrations. (Figure 3)

Twelve rabbits selected at random were maintained in chambers where they were exposed to CO of 50 ppm for twenty-four hours for eight weeks. The other group of six rabbits were maintained in chambers that had the same environmental conditions but no CO passed through. Only normal oil-free air was used as control. Twice a week for eight week period blood samples were drawn from the lateral ear vein of rabbits. Three to four ml of blood were withdrawn into a vacutainer tube (Becton-Dickinson) which contained the anticoagulant and prevented oxygenation of the blocd samples. The vacutainer tubes were tilted gently to achieve adequate mixing. The skin overlaying the vein was cleaned with alcohol or ether.

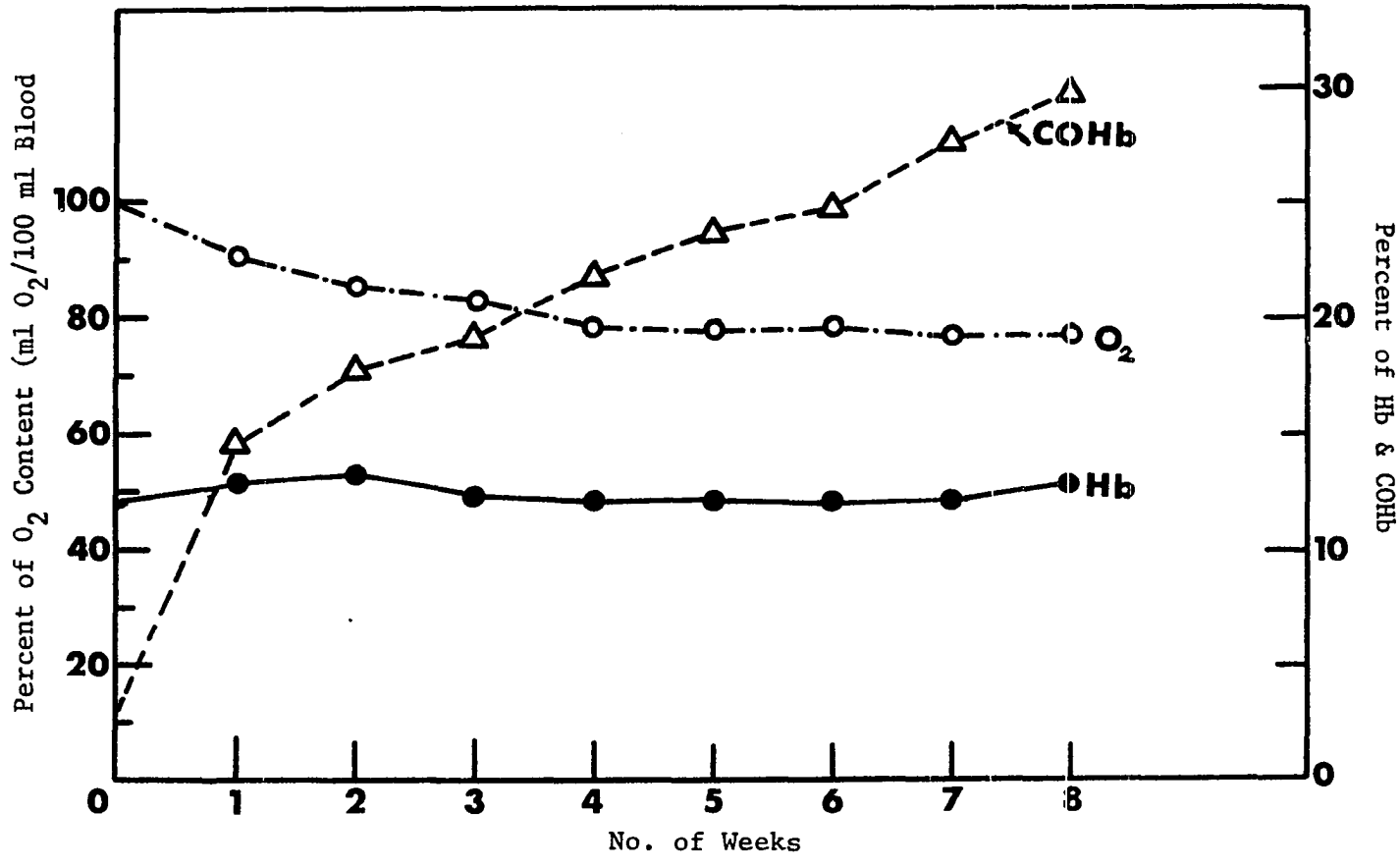
Freshly drawn blood samples were utilized for hemoglobin, carboxy-hemoglobin, oxyhemoglobin and blood fibrinolytic activity which was measured by whole blood clot lysis, a modified Fearnley technique, euglobulin lysis, fibrin plate, caseinolytic assay, fibrinolysis, serum fibrin/fibrinogen degradation products.

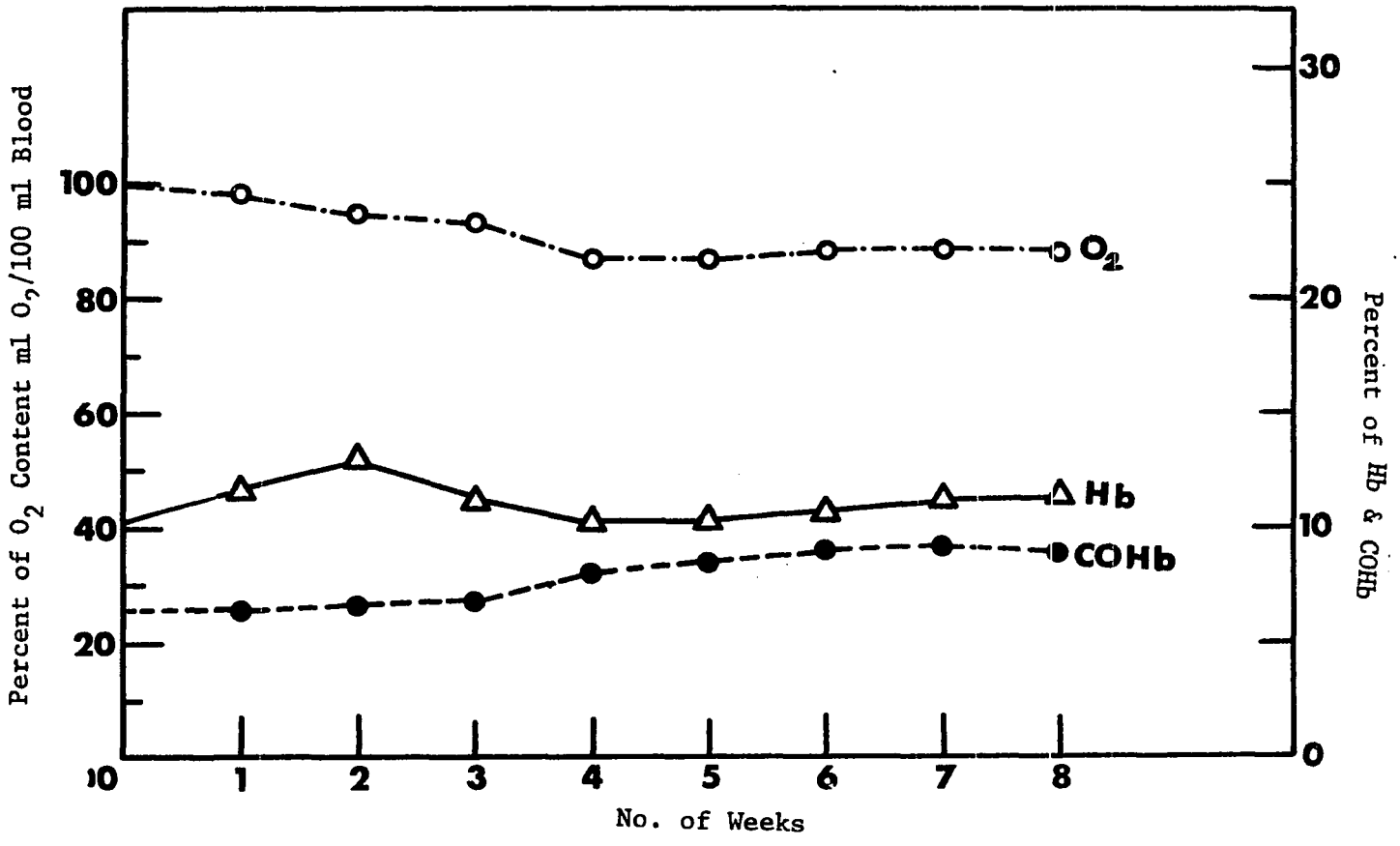
At the end of the 8th week 12 exposed and 6 control rabbits were bled by cardiac puncture and sacrificed by anesthetizing with diethyl ether. The thoracic cavity was opened, and the heart, thoracic aorta were removed and immediately placed in 10% formaline solution for histological examinations.

The instrument processes and analyzes fresh drawn blood samples automatically. A peristaltic pump anaerobically draws up, hemolyzes and moves the sample into the measuring cuvette. Applying the principles

FIGURE 3a. Profiles of mean values of COHb, Hb, O_2. Twelve rabbits (test) that received 50 ppm CO continuously for eight weeks.

b. Profiles of mean values of COHb, Hb, O_2 of six rabbits (control) that maintained under the same environmental condition did not receive CO.


Percent of O_2 Content (ml O_2/100 ml Blood
Percent of Hb & COHb
No. of Weeks
COHb
O_2
Hb
100
80
60
40
20
0
30
20
10
0
1
2
3
4
5
6
7
8


Percent of O_2 Content ml O_2/100 ml Blood
100
80
60
40
20
0
1
2
3
4
5
6
7
8
No. of Weeks
30
20
10
0
Percent of Hb & COHb
O_2
Hb
COHb

of the Beer's Equation in absorption spectroscopy, the instrument simultaneously monitors each blood sample at three carefully selected wavelengths in the green region of the spectrum. (Figure 4)

3.3 Measurement of deoxygenated (or reduced) hemoglobin, oxyhemoglobin and carboxyhemoglobin:

As deoxygenated (or "reduced") hemoglobin, oxyhemoglobin and carboxyhemoglobin each have a unique, but overlapping absorption spectrum, at any given wavelength the total absorbance is a summation of the absorbance of all three species. (Figure 5)

Absorbance measurements were made at wavelengths where the absorptivity of two or more of the species were equal or isosbestic. From the absorbance at 548 nanometers (nm) changes in absorbance were related to changes in total hemoglobin concentration. A change of absorbance at 568 nm compared to the absorbance at 548 nm indicated a change in the relative concentration of COHb. A change at 578 nm relative to 548 nm indicated a change in the relative concentration of oxyhemoglobin. Thus, information was obtained when entered into an electronic computational matrix, allowed presentation of the percent oxyhemoglobin (HbO_2), and the total hemoglobin (Hb) species concentrations presented in digital form COHb and Hb were calculated by solving the following simultaneous equations.

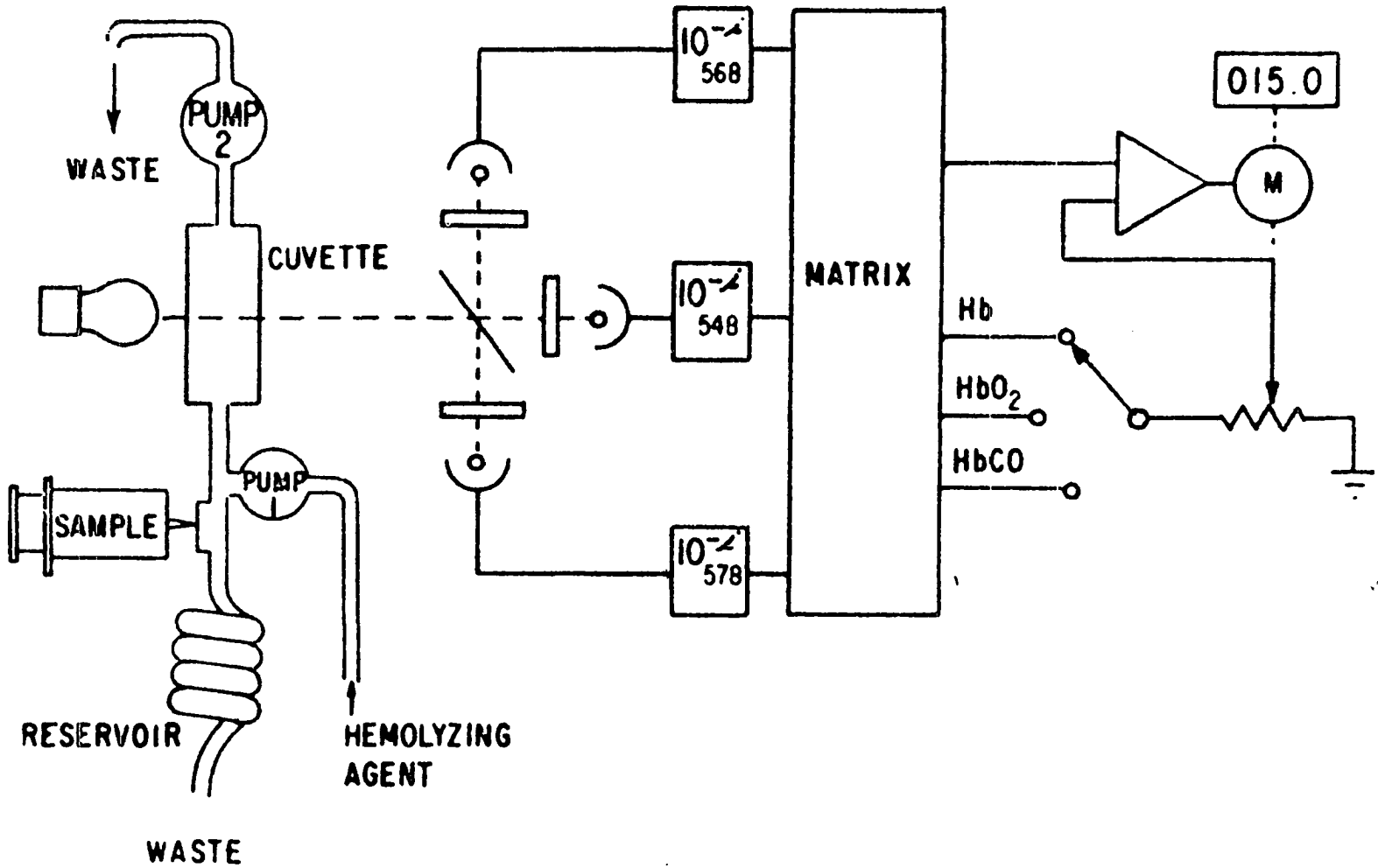
1) $A_1 = a_1 Hbr\ bcHbr + a_1 HbO_2 bcHbO_2 + a_1\ HbCO\ bcHbCO$

2) $A_2 = a_2 Hbr\ bcHbr + a_2 HbO_2\ bcHbO_2 + a_2 HbCO\ bcHbCO$

3) $A_3 = a_3 Hbr\ bcHbr + a_3 HbO_2\ bcHbO_2 + a_3 HbCO\ bcHbCO$

"A" represents the absorbance ($\log I_o/I$) measured at a given wavelength; "a" is the absorptivity of each species at that wavelength, and

FIGURE 4. Sketch Indicating Principle of Operation of the CO-oximeter IL-182.


WASTE
PUMP 2
CUVETTE
SAMPLE
PUMP
RESERVOIR
HEMOLYZING AGENT
WASTE
10^{-1} 568
10^{-1} 548
10^{-2} 578
MATRIX
Hb
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HbCO
015.0
M

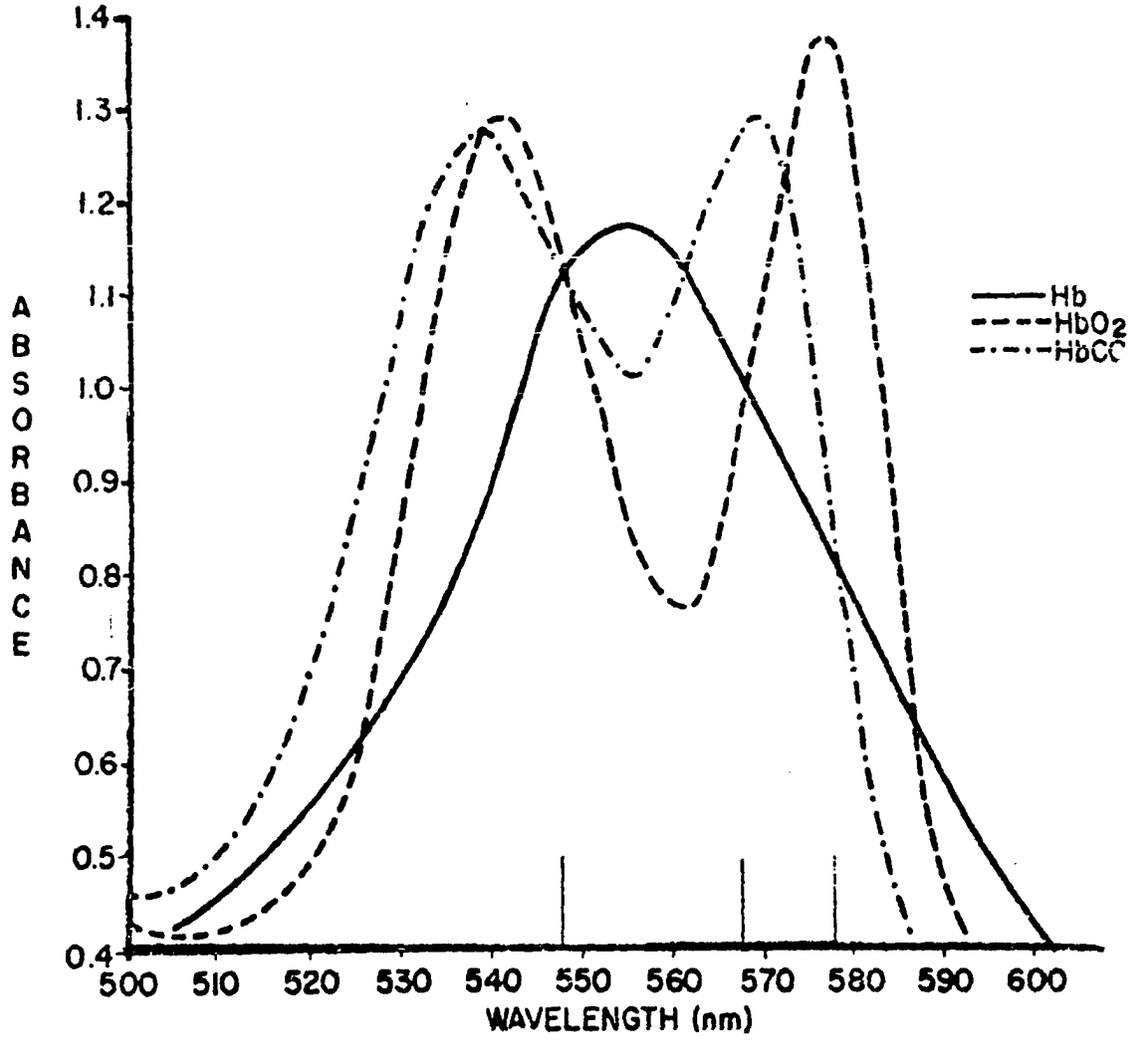


FIGURE 5. Hemoglobin Spectra. Hemoglobin Carboxyhemoglobin and Oxyhemoglobin each have a unique, but overlapping absorption spectrum; at any given wavelength the total absorbance is a summation of the absorbance of all three species.

"b" is the sample path length. 1 = 548 nm, 2 = 568 nm, and 3 = 578 nm.

Equations used to determine percent saturation:

$$Hb = [Hbr] + [HbO_2] + [HbCO]$$

$$\%HbO_2 = \frac{[HbO_2]}{[Hbr] + [HbO_2] + [HbCO]} \times 100$$

$$\%HbCO = \frac{[HbCO]}{[Hbr] + [HbO_2] + [HbCO]} \times 100$$

The Hb species which were measured were deoxygenated, oxygenated, and carboxyhemoglobin. (Table 1 and 2)

3.3.1 Spectrophotometric Determination of Carboxyhemoglobin and Oxyhemoglobin Saturations in Whole Blood:

Freshly drawn blood samples were used in the assay. .02 ml of citrated whole blood was added to 5 ml of 0.1 M ammonium hydroxide. The Beckman DB Spectrophotometer was adjusted at 600 λ with 0.1 M ammonium hydroxide solution then read the optical density of the sample solutions at visible range: (wave lengths: 541, 561, 573, 577, 597).

a) Percent carboxyhemoglobin saturation:

$$\frac{A_1\ 577\ m\mu}{A_2\ 561\ m\mu} = COHb\%$$

b) Percent oxyhemoglobin saturation:

$$\frac{A_1\ 541\ m\mu}{A_2\ 561\ m\mu} = HbO_2\%$$

The results were read from the standard curve shown in Figure 6. The results obtained spectrophotometrically were similar to the results obtained

TABLE 1

12 RABBITS EXPOSED CONTINUOUSLY TO 50 ppm CO FOR 8 WEEKS
(MEAN VALUES OF 8 RABBITS PERCENT OF SATURATIONS OF
Hb, O_2 AND COHb FOR EACH WEEK)

No. of Weeks	Hb, COHb and O_2 Mean Values of Percent Saturations Per Week		
	O_2%	Hb%	COHb%
0	91 - 95.00*	10 - 12**	4.80
1	87.70	11.80	17.60
2	78.10	13.00	19.00
3	75.88	12.65	20.00
4	75.44	12.66	22.50
5	72.50	12.67	24.83
6	72.51	12.74	27.88
7	72.60	12.54	29.94
8	72.71	12.74	30.91

*Normal value: Henson, M., et al., J. Avait M., 18:149 (1947).

**Normal value: Wintrobe, M. M., and Schumacher, H. B., Am. J. Anat. 58:313 (1935).

TABLE 2

6 CONTROL RABBITS (NON-EXPOSED) MAINTAINED IN ENVIRONMENTAL CHAMBERS FOR 8 WEEKS UNDER THE SAME ENVIRONMENTAL CONDITION AS EXPOSED RABBITS BUT DID NOT RECEIVE CO

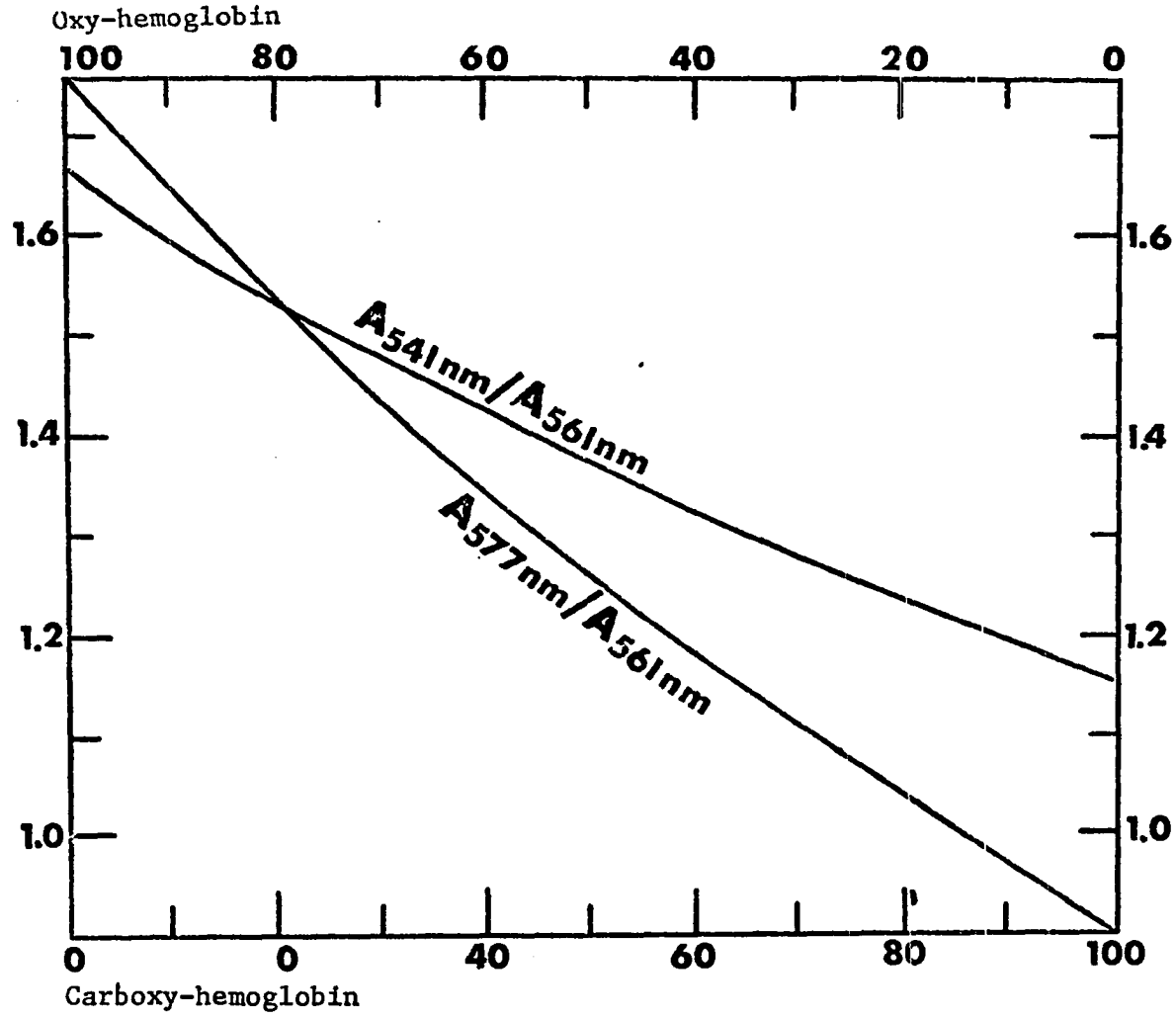
(MEAN VALUES OF 6 RABBITS PERCENT SATURATIONS OF Hb, O_2, AND COHb FOR EACH WEEK)

No. of Weeks	Hb, COHb and O_2 Mean Values of Percent Saturations Per Week		
	O_2%	Hb%	COHb%
0	91 - 95.00*	10 - 12**	4.80
1	95. 00	11.75	5.20
2	89.75	11.62	5.55
3	89.00	12.15	5.20
4	88.50	12.05	5.00
5	89.80	11.85	5.05
6	83.65	12.60	6.70
7	83.55	11.95	7.00
8	83.00	11.85	7.45

*Normal values: Henson, M., et al., J. Avait. M. 18:149 (1947).

**Normal values: Wintrobe, M. M., and Schumacher, H. B., Am. J. Anat. 58: 313 (1935).

FIGURE 6. Standard Graph for Hemoglobin and Oxyhemoglobin determination from spectrophotometric readings.


Oxy-hemoglobin
100
80
60
40
20
0
1.6
1.4
1.2
1.0
A541nm/A561nm
A577nm/A561nm
0
0
40
60
80
100
Carboxy-hemoglobin

from the CO-oximeter. (Figure 3)

3.4 Euglobulin lysis time:

Euglobulin lysis time (ELT), as described by von Kaulla and Schulz (1958): The euglobulin fraction containing fibrinogen, activator and plasminogen, but no antiplasmin, is precipitated by lowering the pH and ionic strength. After redissolving the precipitate and clotting the fibrinogen with thrombin, the clot dissolves spontaneously. This lysis is the result of activation of the plasminogen in the euglobulin fraction by the activator. The resulting lysis time reflects the degree of activator activity present. The technique is as follows:

3.4.1 ELT (Modified Method):

(1) Plasma is separated by centrifugation at 4°C from citrated whole blood immediately on collection.

(2) Acidification to pH 5.9 may be achieved by either (a) adding 9.5 ml. of acidified water (made in large bulk in the ratio of 18.68 ml. of distilled water to 0.32 ml. of 1 percent acetic acid) to 0.5 ml. of plasma; or (b) by adding 15.0 ml. of distilled water to 1.0 ml. of plasma and bubbling a stream of carbon dioxide through this mixture.

(3) After standing at 4°C for 15 minutes, the euglobulin precipitate is collected by centrifugation at 2000 rpm for 5 minutes at 4°C.

(4) The precipitate is redissolved in 0.5 ml. of bovine thrombin solution (10 NIH units per ml.).

(5) The clot is incubated in a water bath at 37°C and observed at frequent intervals for lysis.

Results: Figure 7 gives the representative example of the trend of the ELT of exposed and control groups. There is an increase of fibrin-

FIGURE 7. Decrease in ELT in blood of rabbits that were exposed to 50 ppm CO continuously for eight weeks.

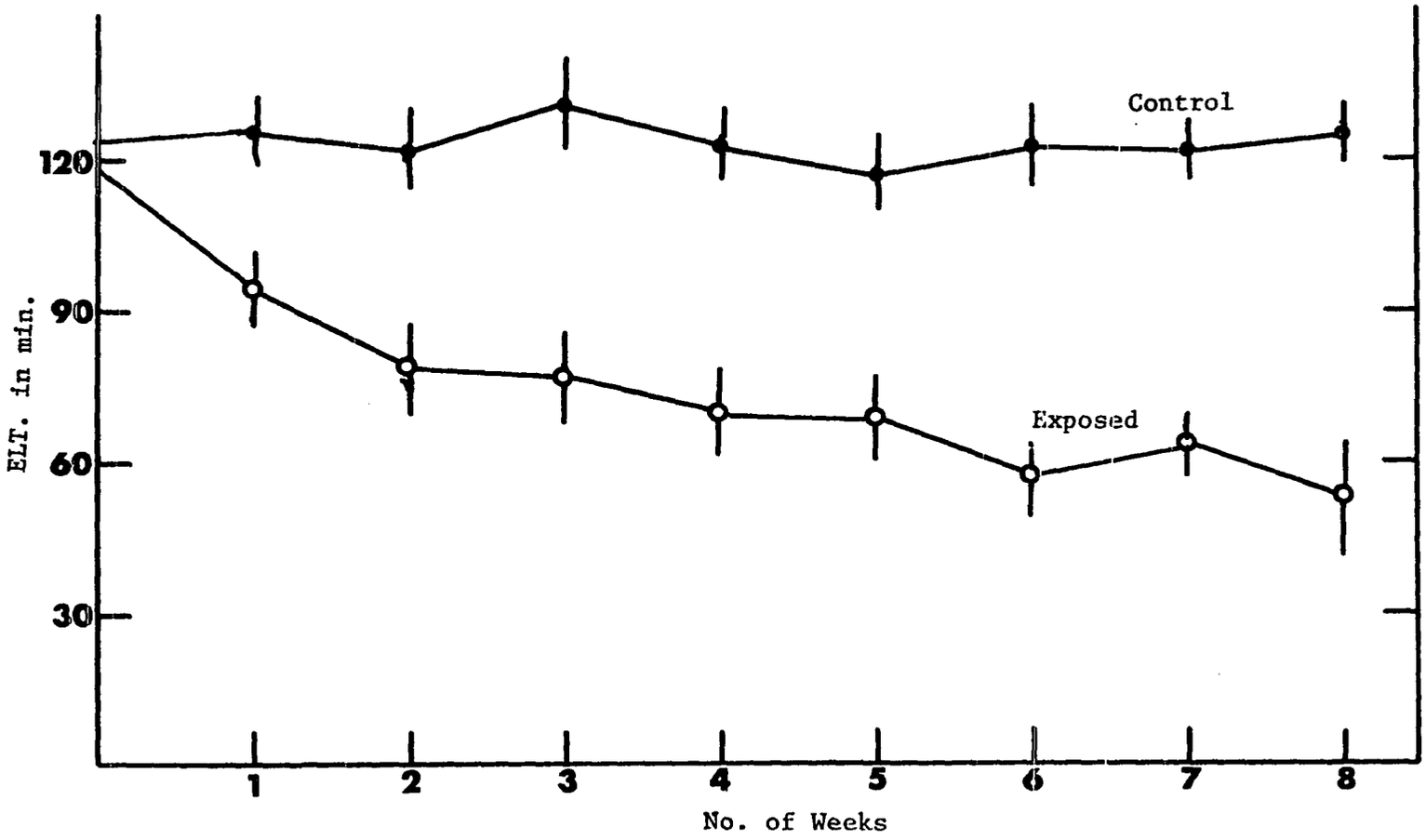

Control
Exposed
120
90
60
30
ELT. in min.
1
2
3
4
5
6
7
8
No. of Weeks

TABLE 3

ELT IN NORMAL-CONTROL RABBITS (48-ELT IN 6 RABBITS, BODY WT. 3.5-4.5 Kgm

ALL MALE MEAN 123. S.D. 30)

1		2		3		4	
No.	ELT (min)	No.	ELT (min)	No.	ELT (min)	No.	ELT (min)
1	104	13	210	25	150	37	110
2	133	14	156	26	125	38	140
3	116	15	120	27	140	39	200
4	158	16	110	28	200	40	160
5	120	17	127	29	180	41	140
6	170	18	160	30	190	42	180
7	141	19	180	31	156	43	210
8	210	20	190	32	135	44	140
9	149	21	200	33	180	45	110
10	120	22	210	34	190	46	130
11	140	23	130	35	160	47	145
12	156	24	156	36	140	48	220

olytic activity after the first week of exposure of CO of 50 ppm. Increase in fibrinolytic activity was related to the concentration of the CO and the time period that CO was exposed.

3.5 Whole Blood Clotting Time:

The technique of whole blood clotting described by Lee and White (1913) was the first attempt at a standardized coagulation assay. The recalcification time was a somewhat more sophisticated technique described by Howell (1916) to detect clotting abnormalities. When the significance of the surface activation was realized, it became obvious that the recalcification time was influenced by contact activation. For this reason, several researchers have attempted to make the test more sensitive by reducing surface activation through the use of either siliconized glass or plasticware. Several improved techniques for improved standardization of the test were developed. In most instances this was accomplished by introducing glass beads and agents with similar surface characteristics to insure consistent maximal surface activation.

The activation of Hageman factor takes place in the presence of substances not present in the vascular system, e.g. silica particles. Activation is also achieved by fatty acids, ellagic acid and collagen (Margolis 1957a; Waaler 1959b; Nossel, 1964c; Hoak, et al., 1967d).

Principle of the method: Blood was collected from the ear of the rabbits. One ml of blood was introduced into each of 3 prewarmed glass tubes and the tubes were placed in the 37°C water bath. The tube one was tilted every 30 seconds until flow of blood stops, leaving the tube two and three untouched. Next tube two was tilted every 30 seconds until completely clotted. At that point, the tilting of tube three was started.

The end point for the test was the time when the blood was clotted in tube three. Clotting time was the total time elapsed from the start of withdrawal of the blood from the vein until completion of clotting in tube 3.

Results: Figure 8 shows the results of whole blood clot time of eight week mean value of test and control groups. The t-test using the eight week mean values of both the test and control groups gave a level of significance of $p > 0.025$ which is a significant difference.

3.6 The whole blood lysis time:

Reagents for the assay - phosphate buffer pH 7.4

Na_2HPO_4 - 9.47 gm in one liter

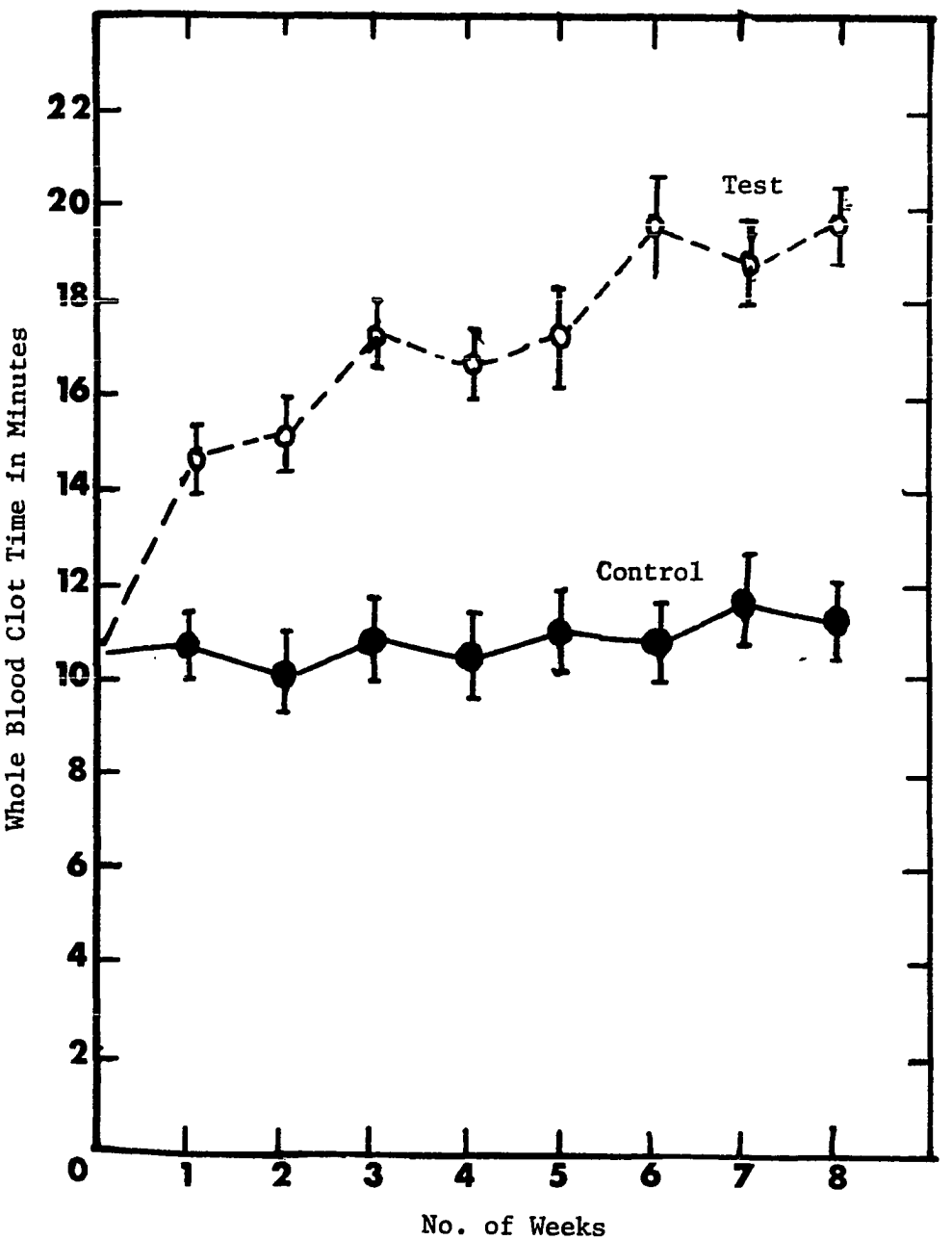
$K H_2PO_4$ - 3.02 gm in 250 ml

Procedure: Ten test tubes were needed. 1.5 ml of blood were taken with siliconized syringe and was added to 5 ml of ice cold buffer and then kept in ice.

Tube No.	1	2	3	4	5	6	7	8	9	10
Blood and Buffer	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.1 ml
Buffer Solution	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1	0.9	0.8 ml
Thrombin Solution	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1 ml

(1) In the first tube put 0.2 ml of the mixture of blood and buffer. In the second put 0.3 ml., in the third 0.4 ml. and so on till the tenth tube put 1.1 ml. of the mixture of blood and buffer as in the above mentioned table.

FIGURE 8. Clotting time is increased in the blood of exposed rabbits (50 ppm CO) as compared to non-exposed rabbit's blood clotting time.


Whole Blood Clot Time in Minutes
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
No. of Weeks
Test
Control

(2) The buffer was added to each of these tubes and made up to 1.9 ml. in each i.e. in the first tube put 1.7 ml., in the second 1.6 ml., in the third put 1.5 ml. and so on till the tenth tube in which 0.8 ml. of buffer was added.

(3) Thrombin solution was prepared by using Parke-Davis.

(4) To each test tube 5 NIH of thrombin was added.

(5) Each tube was gently shaken and left in ice for 15 minutes then transferred to water bath at 37°C for 10 minutes. The tube was rotated gently in order to take the clot off the wall of the tube. The tubes were left in water bath for 12 hours and read the end point which was complete lysis. Then read again after 24 hours.

Results: In the control group 6 subjects (rabbits) blood were examined to eight week period. No fibrinolytic activity could be detected as evidenced by absence of lysing in any of the tubes after incubation for 24 hours at 37°C.

In the test subjects, 12 rabbits' blood were examined for fibrinolytic activity. After incubation at 37°C for 12 hours more than half of the tubes that showed lysis. This ranged from 6-10 with a mean 8.2±1.6. After incubation for 24 hours, the tubes that showed lysis ranged from 8-10 with mean of 9.6±.4. The t-test showed significant variation between the control group and the test group. There was a significant increase in fibrinolytic activity in the blood of exposed rabbits.

3.7 Platelet Morphology:

Blood was collected from the ear of rabbits as described before into a chemically clean dry syringe; care was taken to avoid admixture of

air bubbles. The blood was then rapidly added to test tubes containing the following anticoagulant solutions: 14 per cent magnesium sulfate, 3.2 per cent sodium citrate, 1.6 per cent potassium oxalate, 4 per cent ammonium oxalate, 0.8 per cent potassium axalate with 1.2 per cent ammonium oxalate, 2 per cent sodium oxalate, 0.85 per cent sodium chloride containing enough heparin to provide 100 gamma of heparin (the Upjohn Company, Kalamazoo, Michigan) for each cubic centimeter of blood added. Dilutions of 10:1, 1:1, and 1:10 and 1:100 of blood to anticoagulant solution were made.

From the 1:1 dilutions platelet-rich plasma was removed after one half-hour period of sedimentation or after slow centrifugation, with the exception of the ammonium oxalate solutions which were hemolytic. The anticoagulants used did not affect the pH of the blood significantly in 1:1 dilutions. As measured in the test tube by the glass electrode the pH varied from pH 7.10 to 7.45, and was usually between 7.15 and 7.50.

A small drop of blood or plasma was transferred to a slide and allowed to spread between slide and coverslip. The coverslip was rimmed with vaseline and the preparation was studied with a 97X dark contrast phase oil immersion objective. The 1:100 dilutions of blood to anticoagulant were also examined in a counting chamber with a 43X dark phase contrast objective. The slides were examined from 30 minutes to 12 hours after their preparation.

3.8 Platelet Counting:

Blood was collected in a 10 ml siliconed syringe, through a 20-gauge sharp needle as described before. If bubbling occurred the blood was discarded and new needles and syringes used. One ml. of blood was

drawn into the syringe and the needle was removed. A few drops of blood were placed on a siliconed water glass to facilitate filling of the siliconed red blood cell hemocytometer pipettes. The 20-gauge needle was inserted into the vein, 1 to 2 ml of blood was allowed to flow directly into a siliconed test tube, 10 x 75 mm ,and the pipettes were filled from the test tube. The blood was drawn to the 1.0 mark and then diluted with one percent ammonium oxalate. All of the platelets in 5 blocks of small square were counted after 15 minutes of settling, using a phase microscope, dark contrast 43X objective, long working distant phase condenser, and a counting chamber with an optically flat bottom. Both sides of the chamber were counted, and the total number counted was multiplied by 2500 to give the number of platelets per cu. mm.

Results: Figure 9 shows the results of platelet count in experimental and control rabbits. The t-test, the eight week mean values of both the test and control groups, gave a level of significance of $p > .015$ which is a significant difference.

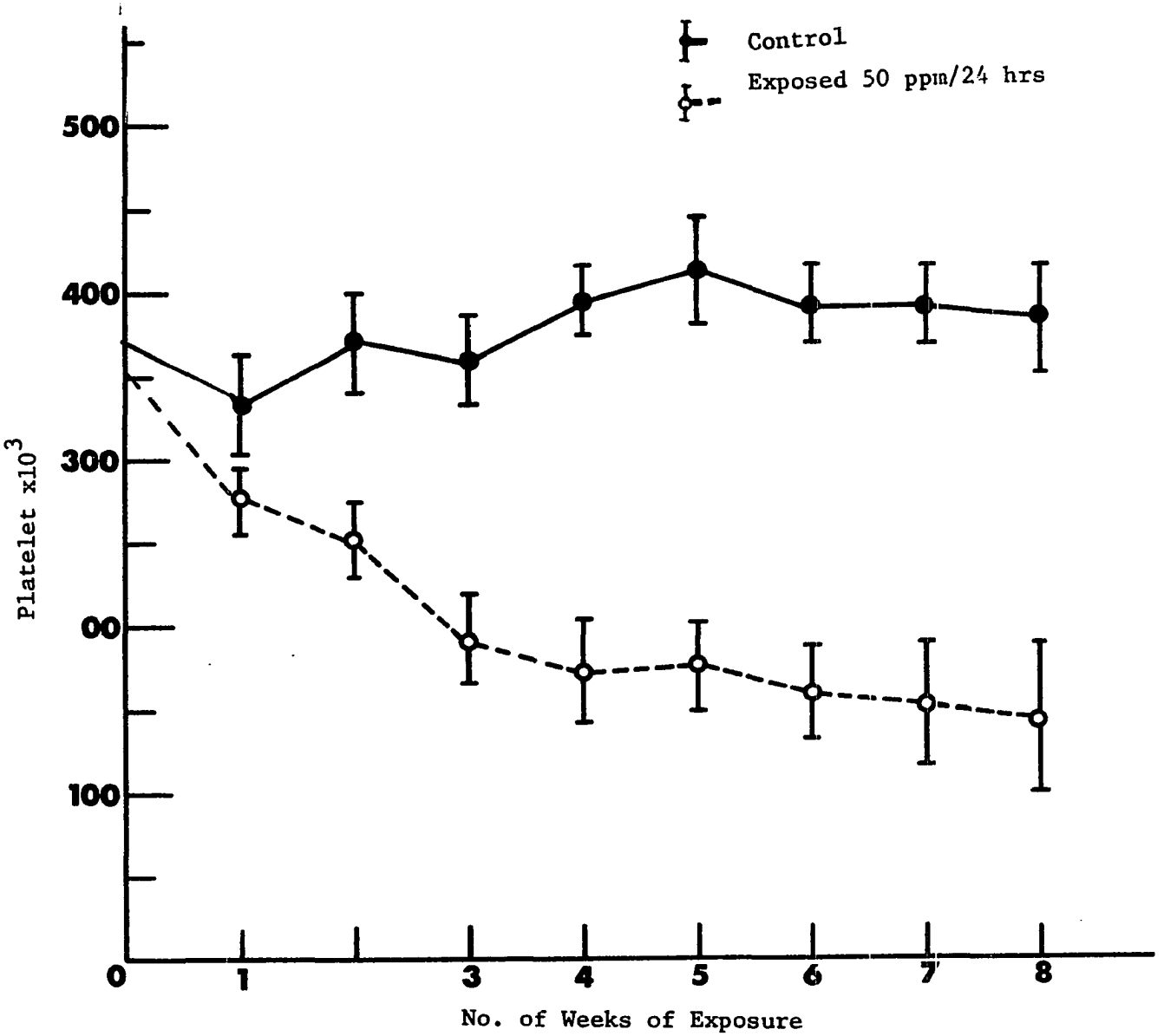
3.9 Isolation of Transglutaminase (Factor XIII) Free of Fibrinogen for Fibrin Plate Preparations:

Pooled human plasma from normal human donors was anticoagulated 1 to 9 with 4% citrate for Cohn Fractionation. The method was used to purify the fibrinogen from human plasma proteins. The method is equally well applicable to human as rabbit plasma. Since proteins are, as a rule, more stable.

3.9.1 Cohn Fractionation:

The method was used to purify the fibrinogen from plasma proteins. The method was equally applicable to human as well as rabbit plasma.

FIGURE 9. Platelet quantity is reduced in the blood of exposed rabbits compared with the non-exposed rabbit's blood. By comparing the decrease of number of platelets in exposed rabbits blood with the control (non-exposed rabbits) blood, shows evidence of vascular platelet consumption or platelet destruction in the circulation of exposed rabbits blood.


Control
Exposed 50 ppm/24 hrs
500
400
300
00
100
0
1
3
4
5
6
7
8
Platelet x10^3
No. of Weeks of Exposure

Since proteins are, as a rule, more stable in the solid state, and dissolution of fibrinogen in the presence of prothrombin had to be involved undue risks, in purification of fibrinogen as far as possible by extracting the contaminants.

Dissociation of the protein complexes in fraction I with simultaneous quantitative separation of the components was, however, difficult to achieve by simple extraction with buffer solutions of varying pH and ionic strength. Preliminary fractionations nevertheless showed that separation was highly effective when extraction was performed in the presence of glycine. Glycine in high concentration enhanced the salting-out effect on the fibrinogen, where the salting-in on the contaminating proteins were considerable. Under these conditions, 95% of the fibrinogen was recovered in the residue (fraction I-0), whereas the major part of the impurities were extracted. With this procedure, the coagulability of the protein increased from 40 to 50% in fraction I to 85 to 90% in fraction I-0.

Fractionation procedure: At first plasma centrifuged 4000 revolutions per minute (RPM) for 30 minutes by Sorvall Model RC-3 centrifuge. The small amount of red cell precipitate was removed from the plasma. The fractionation was done in the cold bath (Fisher Serological Bath). Ethanol chilled ($0^{o}C$) was added to the plasma (initial concentration of ethanol 53% v/v to the final concentration of 8% v/v). pH was adjusted to 7.4 by adding 0.8 M sodium acetate. Small motor stirrer, was stirring constantly during the addition of ethanol one and half hour of time given for the total volume of ethanol to be added. During the addition of ethanol pH was checked frequently in order to be sure that pH of the plasma was kept around 7.2 (pH was adjusted with 7% acetic acid). During

the addition of ethanol the temperature of the system was kept below 0°C close to -2°C so that the system was maintained close to the freezing point. The final temperature was -3°C. After one and half hours, the reaction mixture was centrifuged at 2500 RPM for 20 minutes at -3°C. The supernatant which was rich in plasminogen was frozen at -20 and saved for further purification. Precipitate was washed with chilled amino-acid citrate buffer pH 6 and 7 (75 g. glycine, 65 ml absolute ethanol, and .055 M sodium citrate made up to 1 liter and pH was adjusted with 6N hydrochloric acid).

First wash was with the pH 6 buffer (used 1/4 of the original volume of the plasma). The buffer was added with caution and constant stirring for 1 hour at -3°C was done. The mixture was centrifuged at 2500 RPM for 20 minutes and the supernatant was discarded.

Second wash was with the pH 7 buffer (used 1/4 of the original volume of the plasma). The second buffer was added with caution and constant stirring for 1 hour at -3°C was made. The mixture was centrifuged at 2500 RPM for 20 minutes and the supernatant was discarded. After second wash the pellet was dissolved in .05 M sodium citrate buffer pH 6.35 (the vol. of the buffer was 1/8 the original vol. of plasma) and kept at 30°C for 30 minutes, then diluted to final concentration of 1.5% with .05 M sodium citrate buffer. This diluted Cohn Fraction I-O fibrinogen was further purified by column chromatography.

Chromatography: Cohn Fraction I-O solution containing 250 mg of protein were diluted to 10 ml with "starting buffer" and dialyzed in the cold room, 4°C for 48 to 72 hours against four changes, approximately 750 ml each, of the same buffer.

"Starting buffer" (pH 8.6) was .005 M in H_3PO_4 and 0.029 M in Tris (hydroxymethyl)-amino-methane(Tris). The final buffer was 0.5 M in both H_3PO_4 and Tris, and had a pH of 4.1. After dialysis the Cohn Fraction I-O sample was placed on a 2.2 cm x 30 cm column of diethyl-aminoethyl) (DEAE) cellulose equilibrated with starting buffer. The DEAE cellulose, (Scheicher and Schuell, Inc.) equilibrated with starting buffer. The chromatography was done at $4^{o}C$, essentially by the method of Finlayson (1963). A compound concave elution gradient was obtained with a nine-chambered gradient elution device ("Varigrad") of the type described by Peterson (1958). Solutions placed in the chambers consisted of various amounts of final buffer diluted to 220 ml with starting buffer. The volumes per cent of final buffer in successive chambers were 0, 1.6, 2.0, 9.0, 9.0, 20, 20, 100, and 100. A flow rate of 36 ml per hour was used and 8-ml fractions were collected (Figure 10). The fractions were read at 280 mμ in a Beckman DB spectrophotometer. Fractions containing pure fibrinogen were pooled and dialyzed against .05 M sodium citrate solution pH 6.3 then lyophilized and the purity of the fibrinogen subfraction was checked by 6% polyacrylamide gel electrophoresis and immunoelectrophoresis. This concentrated chromatographic subfraction was found to be contaminated with Factor XIII. Rechromatography of this fibrinogen subfraction was done on a column of Whatman DE-23 (diethyl-aminoethyl cellulose) from W. and R. Balson Ltd. England.

Same Tris-phosphate buffer system with compound concave elution gradient was used. This rechromatographed fibrinogen subfraction showed a single band on 6% polyacrylamide gel electrophoresis, immunoelectrophoresis, and produced an acetic acid-soluble clot (Figure 11). The assay

FIGURE 10. Chromatography of fibrinogen (Cohn Fraction I-O). The profile shows the elution pattern of the fibrinogen of DEAE column in the cold room. Clottable protein is indicated by the dashed line.
▬▬ Phosphate salts molarity.
●-● pH of elution.

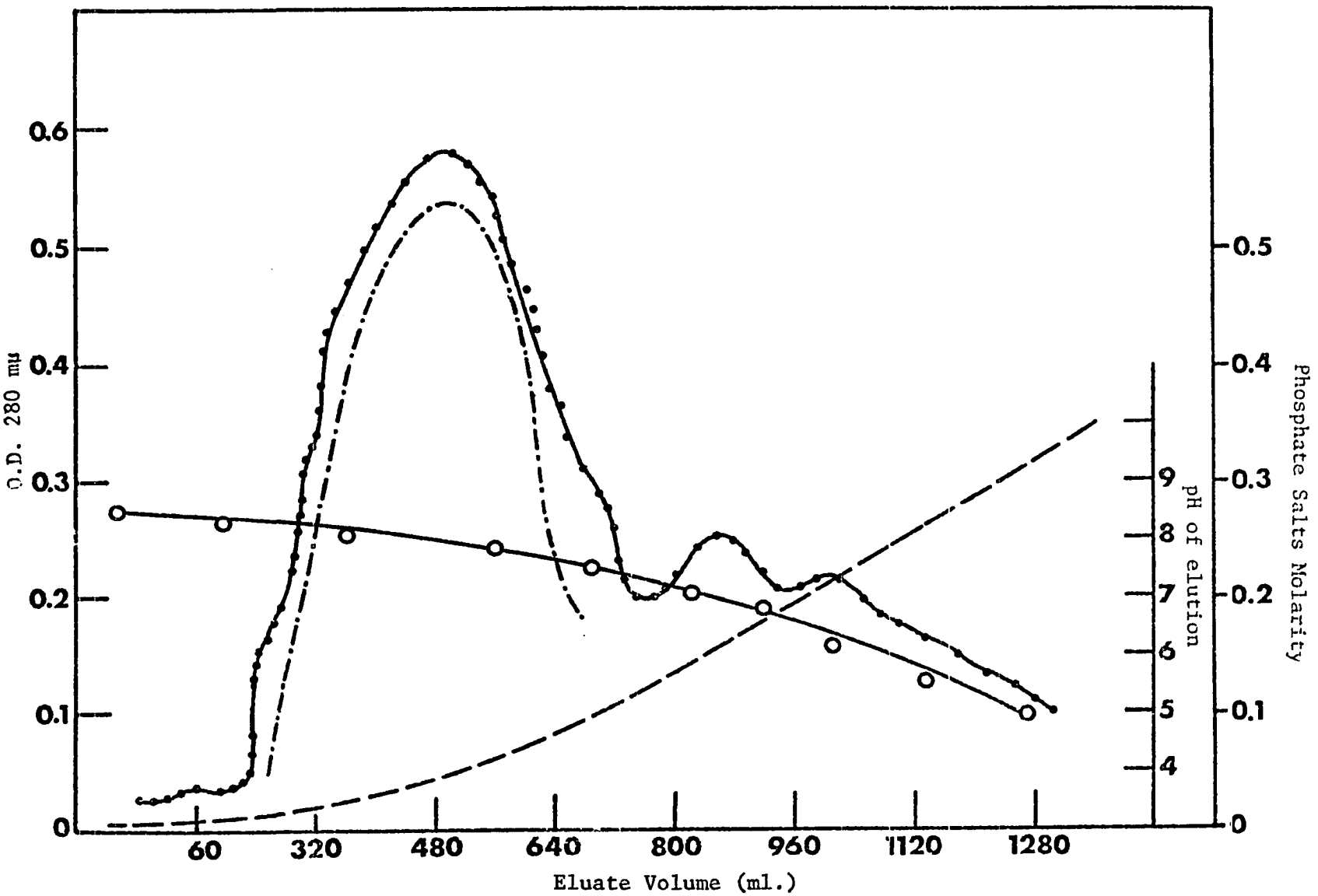

O.D. 280 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0
60
320
480
640
800
960
1120
1280
Eluate Volume (ml.)
pH of elution
9
8
7
6
5
4
0.5
0.4
0.3
0.2
0.1
0
Phosphate Salts Molarity

FIGURE 11. Immunoelectrophoresis of purified fibrinogen and rabbit anti-human fibrinogen antiserum. The wells were filled with 1 µl of fibrinogen at 10 mg per ml, and migration proceeded for 90 minutes at 250 volts, the troughs were filled with rabbit anti-human fibrinogen antiserum. Wells A and B are purified (Factor XIII) free fibrinogen.

for transglutamirase did not show any transglutaminase activity. The fibrinogen subfraction free from Factor XIII was used for the fibrin plate preparations.

3.10 Preparation of Fibrin Plate:

The method described by Astrup and Müllertz was used for preparation of fibrin plates. Plasminogen rich fibrinogen, required for making fibrin plates, was prepared as follows: Human plasma was diluted with 0.5 vol. cold distilled water followed by 0.6 vol. of cold saturated ammonium sulfate, was slowly added in the cold room. The precipitate was redissolved in 0.5 vol. of saline and diluted with one vol. cold distilled water. The fibrinogen solution obtained was again precipitated in the cold room by slowly adding 0.6 vol. of cold saturated ammonium sulfate. The precipitate was next dissolved in 0.2 vol. distilled water, the pH was adjusted to 7.5 and the ionic strength to 0.3 or 0.4 by measuring electrical conductivity in reference to a standard sodium chloride solution (sodium chloride in solution was assumed to be completely ionized). This fibrinogen-plasminogen solution which usually had a fibrinogen concentration between 1.5-1.8 gm percent was then stored frozen in 10-15 ml lots at $-20^{o}C$.

Fibrin plate buffer, pH 7.75, ionic strength 0.15 was used to prepare the fibrinogen solution was made by mixing 20.62 gm sodium barbital, 1350 ml distilled water, 500 ml 0.1 N HCl and 100 ml 0.1 salt solution (see below) in a 2000 ml vol. flask. The pH was adjusted to 7.75 with 0.1 N HCl; and the solution made up to 2 litres with distilled water.

The salt solution was prepared by mixing 4.891 gm $CaCl_2 \cdot 2H_2O$, 2.787 gm $MgCl_2 \cdot 6H_2O$, 109.12 gm NaCl with distilled water to make a litre.

Petri dishes (Optilux, 100x15 Falcon Plastics) were used to measure fibrinolytic activity. Unheated plates were prepared from the frozen fibrinogen which had been thawed slowly at room temperature, centrifuged at approximately 2000 G for thirty minutes and filtered through cotton wool. It was then diluted to a concentration of 0.1 percent and the ionic strength was adjusted to 0.15 with the "fibrin plate" buffer. Each petri-dish was filled with 6 ml of 0.1 percent fibrinogen solution and the fibrinogen was clotted by adding 0.2 ml thrombin solution (20 units per ml). The fibrinogen and the thrombin were carefully mixed by moving the plate in a horizontal plane with a perpendicular motion. Then the plate was left for one hour at room temperature in a perfectly horizontal position. The plate was ready for use after incubation at 37°C for 20 minutes. Three hundredths ml of the unknown solution to be assayed for fibrinolytic activity was placed carefully on the surface of the fibrin plate. Each unknown is tested in duplicate or triplicate on the same plate. The plate was then incubated at 37°C for 16 to 18 hours. The diameter (D) of the lysis area (a clear round zone liquid surrounded by undigested fibrin) was measured in mm on two axis, perpendicular to each other. The product of the two diameters as the measure of the lysis area was expressed as sq. mm which, although not representing the actual lysis surface, area, was an accepted way of expressing the results.

The fibrinogen solution was plasminogen-rich. Plasminogen co-precipitates with fibrinogen, and its concentration will be directly related to the fibrinogen concentration. If plasminogen is present in the small volume of only 0.03 ml of the testing solution, it will be very little compared to the amount of plasminogen already contained

within the fibrin plate. Consequently, plasminogen contamination of the solution to be tested has little effect on the results. The lysis area of the unheated fibrin plate, indirectly reflects but allows for measurement of the fibrinolytic activity.

To measure plasmin activity the heated plate (Lassen, 1953) was used. The heated plate was prepared in a manner similar to the unheated plate with the exception that a flat bottom, glass Petri dish was used. After 20 minutes incubation at 37°C, the plates were transferred into an oven with a temperature setting between 85-90°C, and were heated at this temperature for 30 minutes in order to denature all the plasminogen contained within the fibrin clot. After cooling down the room temperature, the heated fibrin plates were pre-incubated at 37°C before use. The solution to be tested were applied as previously described for unheated plates and the results were expressed in a similar fashion. Since all the plasminogen was denatured by the heat, any lysis area produced represented the activity of plasmin or any other proteolytic enzyme which could digest fibrin.

3.10.1 Fibrin Plate Test (HYLAND)

Fibrin plate test is used to examine three components of the fibrinolytic system: available plasmin, active plasmin, and total plasminogen. Fibrin plate test is a sensitive and accurate indicator of fibrinolysis in plasma or plasma preparations.

The fibrin plate consists of a buffered agarose gel containing a homogeneous fibrin clot which is Factor XIII free and essentially free of plasminogen. Plasma or a plasma preparation placed in a well in the agar gel diffuses into the agar and lyses the fibrin clot, forming a clear

reaction zone. The zone diameter is directly proportional to the log percent concentration of available fibrinolytic enzyme in the sample. Each fibrin plate contains six wells: for both available plasmin and active plasmin tests this is sufficient to determine activity in two samples; for the assay of total plasminogen one plate is used to prepare a reference curve and simultaneously to test one unknown sample.

3.10.2 Available Plasmin Test

(1) At first the plate was opened, and set in room temperature for 15 minutes.

(2) Added 0.5 ml of citrated test plasma to 0.05 ml of streptokinase. Incubated 10 minutes at 37°C.

(3) Three wells of the fibrin plate were filled with the incubated mixture. Touched the tip of the capillary to the bottom of the well and allowed a drop of the solution to flow onto the base of the plate.

The capillary moved in a circular motion on the base plate until the bottom of the well was completely covered with solution, then continued filling the well until it is no longer visible when viewed from an angle.

(4) The top cover of the plate was replaced and incubated at 37°C for 8 hours.

(5) The diameter of the lysis zone was measured to the nearest 0.1 mm. Then the diameters were averaged.

Results: Clear zone demonstrated the lysis area on unheated fibrin plates induced by the rabbit plasma that exposed to CO of 50 ppm. The elevated fibrinolytic activity was measured after the second week of exposure. It appears as a clear, lysed, nearly round zone, surrounded

by solid fibrin clot. Two perpendicular diameters (D) were read, in mm. Lysis areas were calculated by DxD. The size of each lysis area produced by plasma was converted in CTA units of urokinase by reading from the urokinase standard curve. Each plasma sample was tested on five fibrin plates and applied in triplicate on each plate. Some variation in measurement was found both on the same fibrin plate and also between the different fibrin plate. The most obvious causes of variation in this measurement, that was, lysis areas which were not completely round. Theoretically, the lysis areas should be round as in Figure 14. Although the incubator was always carefully levelled, the lysis areas were not always completely round.

3.11 Purification of Plasminogen for the Caseinolytic Assay:

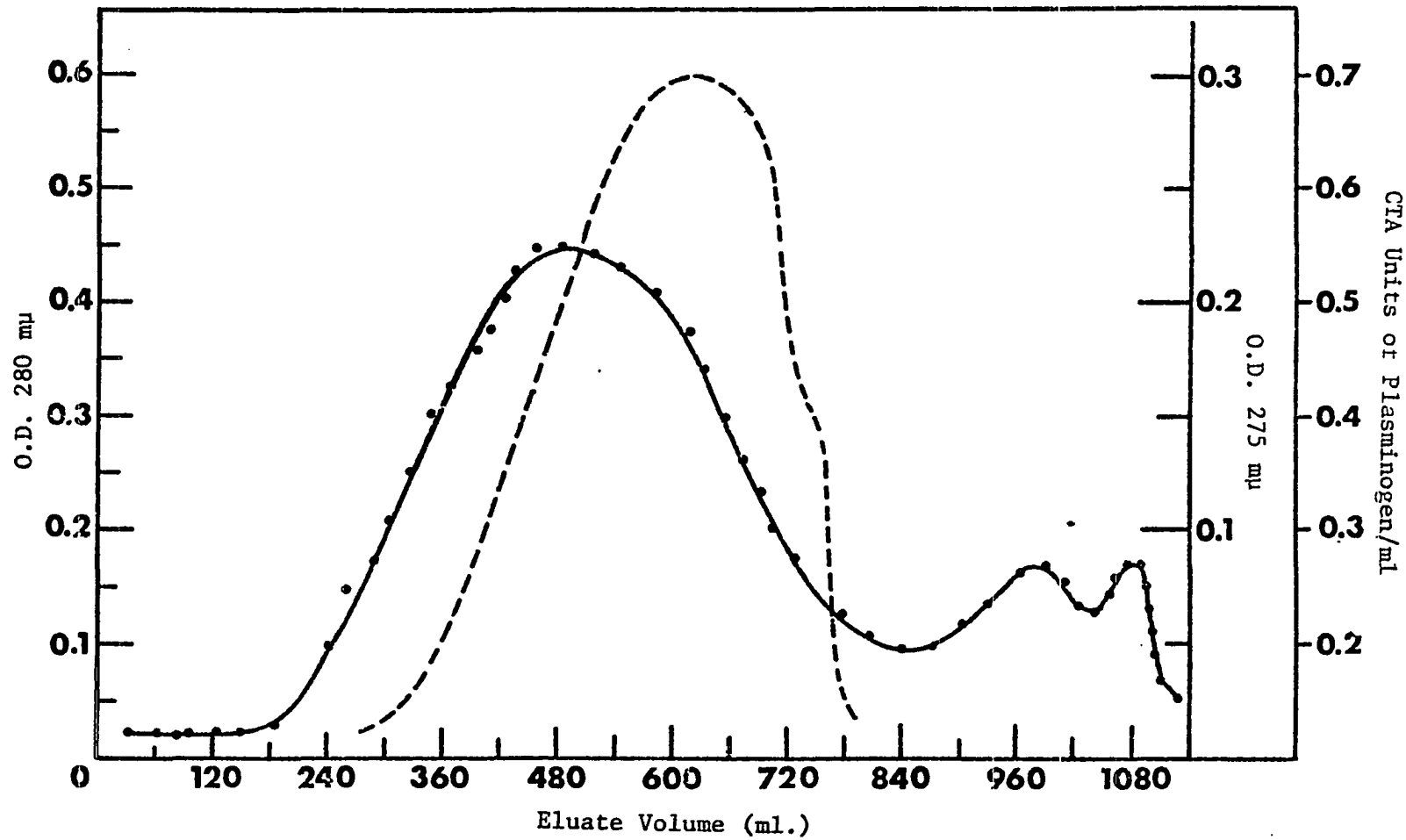
Plasminogen was prepared from human plasma by affinity chromatography on L-lysine-substituted sepharose. Thirty milligrams of plasminogen, with a specific activity of 100 caseinolytic units (Committee on Thrombolytic Agents) per milligram of nitrogen, were obtained from 400 ml of plasma. This corresponds to over 200-fold purification from plasma. Plasminogen is the zymogen of the proteolytic enzyme plasmin (Alkjaersig, 1964a; Cuatrecases, et al., 1968b). The enzyme responsible for the dissolution of fibrin clots in the blood. In a continuing effort to resolve problems of activity, yield, reproducibility, homogeneity, stability, and solubility at physiological pH, dozens of methods have been used for the preparation of plasminogen (Remmert, et al., 1949a; Kline, et al., 1961b; Slotta, et al., 1964c; Robbins, et al., 1965d; Chan, et al., 1966e). In purification of plasminogen ε-aminocaproic acid (ε-ACA), and

inhibitor of plasminogen activation to plasmin by covalently binding the α-amino group of L-lysine to sepharose. Preparation of plasminogen by affinity chromatography, is an attractive alternative to the multistep procedures used before.

Agarose (Sepharose 4B, Pharmacia) was activated with cyanogen bromide (Cuatrecasas, et al., 1968). To a 150-ml suspension (0.1 M $NaHCO_3$ buffer, pH 8.9), containing 100 ml of activated agarose, 20.0 g of L-lysine monohydrochloride was added, titrated to pH 8.9 in 50 ml of water and slurry was stirred for 24 hours at 4°C. The amount of lysine bound to the agarose was 55 micromole (μ mole) per milliliter of settled agarose, determined by Deutsch. If one assumes the molecular weight of plasminogen is 81,000 then each ml of settled lysin-sepharose could theoretically bind about 4 g of plasminogen provided that all the lysine molecules joined to the sepharose were available for plasminogen binding and a 1:1 stoichiometry exists between enzyme and inhibitor.

Agarose column (2.5 cm x 30 cm) containing 50 ml of degassed lysine-sepharose was equilibrated with 0.1 M phosphate buffer (pH 7.4). A volume of 400 ml of plasma was diluted to 600 ml with water and passed through the lysine-sepharose column at 75 ml per hour and the column was next washed with 0.1 M phosphate (pH 7.4) at 175 ml per hour until the absorbancy at 280 nm was less than 0.01. The plasminogen was then eluted as a sharp peak with 0.2 M ε-aminocaproic acid in 0.1 M phosphate buffers (pH 7.4) at 100 ml per hour (Figure 12). All of the steps in the procedure, up to this point were performed at 4°C, and .003 M EDTA (ethylene diamintetraacetate) was added to the phosphate buffer as well as to the plasma.

FIGURE 12. Affinity chromatography of a plasminogen by elution of 0.2M ε-aminocaproic acid with 0.1M phosphate buffer pH 7.4. The dotted peak shows the caseinolytic assay of plasminogen activity.
▬ protein peak
▬ ▬ activity peak


0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. 280 mμ
0.3
0.2
0.1
O.D. 275 mμ
0.7
0.6
0.5
0.4
0.3
0.2
CTA Units or Plasminogen/ml
120
240
360
480
600
720
840
960
1080
Eluate Volume (ml.)

The ε-aminocaproic acid was removed from the plasminogen in the cold, by dialyzing against buffer (.06 M Tris, 0.09 M NaCl, 0.02 M Lysine) pH 8.9 overnight.

Plasminogen was freeze-dried and stored at -20°C. Plasminogen activity was measured by a standard caseinolytic assay suggested by the National Heart Institute's Committee on Thrombolytic Agents (CTA), Subcommittee for standardization with the specific activity expressed in CTA casein units per optical density unit. When 250 plough units of urokinase were used for activation of plasminogen to plasmin and when the plasminogen concentration was measured at 280 nm, the affinity chromatography preparation had an average specific activity of 10 CTA units per absorbency unit (100 CTA units per ml of nitrogen) with no detectable spontaneous plasmin activity under the condition of the assay.

3.11.1 Caseinolytic Assay

0.3 ml of sample solution (plasma) was added to 1 ml plasminogen solution (8.5 CTA units/ml). The tubes were incubated for 10 minutes at 37°C then 2.5 ml of 1.4% α-casein solution was added. After 2 minutes and 32 minutes incubation, respectively a 2 ml aliquot was removed and added to 3 ml of 0.5 M perchloric acid, and the content was mixed gently for a few seconds. The precipitates were left at room temperature for 30 minutes. The perchloric acid precipitates were removed by filtration (Munktell paper No. 00) into tubes for optical density (OD) measurements. The optical densities of the filtrates were read at 275 mμ. The difference in OD between the 30 minutes and zero time samples was the activation of plasminogen by the sample solution.

Results:

The t-tests were used as a test for significance between eight week mean values of both the test and control groups. The results of these tests were highly significant with $P > .001$.

3.12 Standard Urokinase Curve:

A series of urokinase dilutions was prepared (1, 2, 3, 4 and 5 CTA unit/ml gelatin buffer. One ml aliquots of each urokinase (UK) dilution were mixed with 1 ml plasminogen solution and 0.5 ml buffer. A blank was made by substituting 1 ml gelatin buffer for the UK. The tubes were incubated for 10 minutes at 37°C then 2.5 ml of 1.4% α-casein solution was added. After 2 minutes and 32 minutes incubation, respectively, a 2 ml aliquot was removed and added to 3 ml of 0.5 M perchloric acid. The tubes were treated exactly as the caseinolytic assay of plasmin or plasminogen. The blank value, representing spontaneous plasmin activity in the plasminogen preparation, were subtracted from the reading obtained in the tubes containing urokinase. The standard curve and the mean from 15 assays are shown (Figure 13).

Reagents:

(1) Gelatin buffer: 10 g purified calfskin gelatin (Eastman Organic Chemicals, Rochester, N.Y.) dissolved in 1 litre of phosphate, or tris, or Tes buffer, placed in a boiling water bath for 1 hour or until the gelatin was dissolved and filtered, and kept frozen in small portions.

(2) Perchloric acid: (0.5 M) for caseinolytic assay: 42.8 ml of 70% perchloric acid made up to 1 titre with distilled water.

(3) Phosphate buffer: (0.06 M) - NaCl (0.09 M) buffer: 8.517 g Na_2HPO_4 (anhydrous) and 5.26 g sodium chloride dissolved in 900 ml distilled water, pH adjusted to 7.5 with N hydrochloric acid and the volume made up to 1 litre.

(4) α-casein: 1.4 gram α-casein dissolved in 100 ml of tris-NaCl buffer.

(5) Plasminogen: a solution containing 8.5 CTA plasminogen units/ml buffer.

(6) TES-NaCl buffer (N-tris(hydroxymethyl)methyl-2-amino-ethane solfonic acid).

3.12.1 Urokinase determination in rabbit urine:

Urine (3-4 ml) is first dialyzed in cellulose dialysis tubing (Union Carbide O.D. 7 mm) against buffer saline pH 7.4 for 6-10 hours at 4°C. The fibrin plate technique was used for measuring urokinase activity in dialyzed undiluted urine samples. The urokinase assay in each urine sample was run in triplicate.

After (3-4 ml.) were removed for measuring urokinase activity in fresh urine, the individual urine samples were pooled and kept refrigerated until 24 hours collection were completed. Urokinase activity was then measured in the 24-hour urine volume.

Figure 14 demonstrates the lysis area on unheated plates induced by urokinase in exposed and non-exposed rabbit urine, using the standard amount of 0.1 ml urine. It appears as a clear, lysed, nearly round zone, surrounded by solid fibrin clot. Two perpendicular diameters (D) are read, in Figure in mm. Lysis areas were calculated by DxD. The size of each lysis area produced by ruine is converted in CTA* units of uro-

*Committee of Thrombolytic Agents of the National Heart Institute.

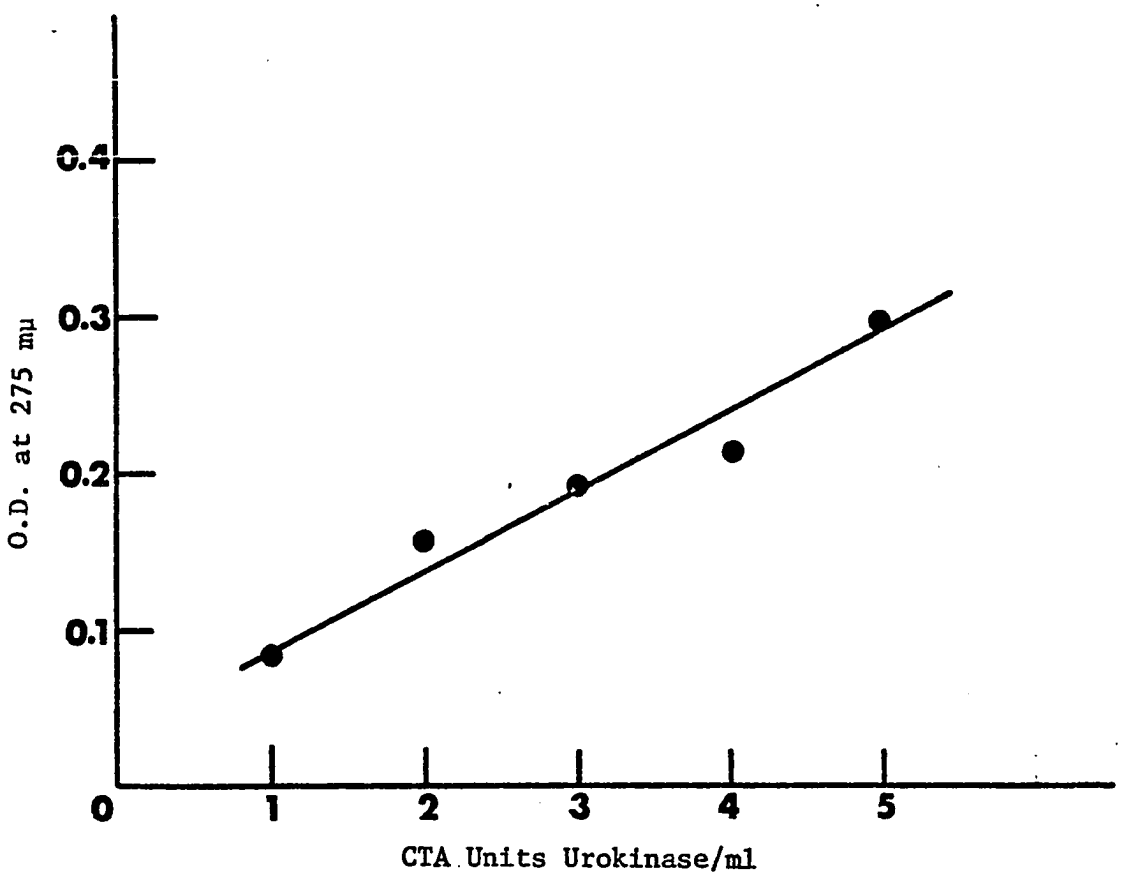
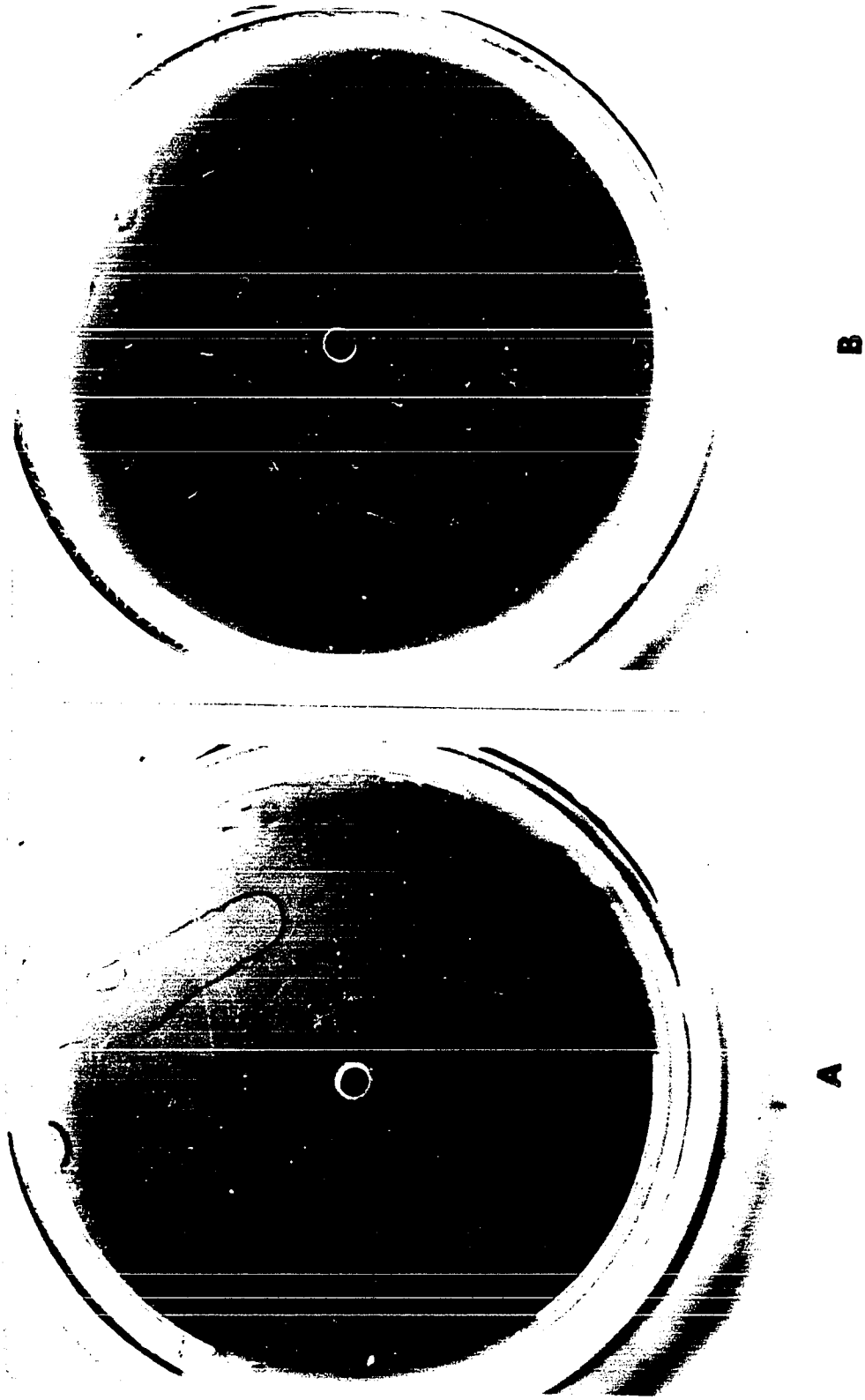


FIGURE 13. Urokinase Standard Curve: Ordinate readings are optical density at 275 mμ. Abscissa readings are CTA units of urokinase used for activation of plasminogen to plasmin in the caseinolytic assay.

FIGURE 14. Example of lysis area produced on unheated fibrin plates by 0.1 ml dialyzed undiluted rabbit urine.

A - Control (non-exposed)

B - Test (Exposed to 50 ppm CO)


B
A

kinase by reading from the urokinase standard curve.

The reproducibility of fibrin plate method. Table 4 illustrates the results of fibrinolytic activity--measured as lysis area--of 3 different urine samples (A,B,C). Each urine sample was tested on five fibrin plates and applied in triplicate on each plate. Some variation in measurement was found both on the same fibrin plate and also between the different fibrin plates. The range of the variation in the lysis area among 3 observations taken from the mean on each fibrin plate, a total of 15 fibrin plates, was ± 5.0% to 24.2% (calculated from the range of 2 S.D.) with an average of ± 10.2%. The difference between lysis areas on the fibrin plates of ± 10.2% would have to be considered as insignificant.

The most obvious causes of variation in this measurement, that is, lysis areas which are not completely round. Theoretically, the lysis areas should be round as in Figure 14. Although the incubator is always carefully levelled, the lysis areas are not always completely round. However, the lysis areas on which the urokinase standard curve (to be described in the following section) is based, has the same range of variation as the ones caused by the urine samples.

3.12.2 Urokinase standard curve:

This was prepared as follows: Urokinase (calbiochem) was used as a standard urokinase. This urokinase is serially diluted in 1% glycerol buffer saline pH 7.4 (glycerol added to preserve urokinase activity after dilution) to arrive at urokinase concentrations from 0.025 to 60 CTA units/ml. Each concentration is applied in triplicate on untreated fibrin plates. The mean lysis area (mm^2) produced by each urokinase concentration after 18 hours of incubation is calculated, and then plotted

TABLE 4

No. of Urine Sample	No. of Fibrin Plate	No. of Measurement on Each Fibrin Plate	Lysis Area (mm^2) on Unheated Fibrin Plate				
			I	II	III	Mean ± S.D.	% Deviation From Mean (2S.D.)
A	1	3	576	624	621	607 ± 27	9.0
	2	3	625	552	552	526 ± 42	14.6
	3	3	625	650	624	633 ± 15	5.0
	4	3	625	600	576	600 ± 24	8.0
	5	3	529	600	600	576 ± 40	14.0
B	6	3	672	550	546	589 ± 70	24.2
	7	3	600	552	575	575 ± 24	8.0
	8	3	625	576	600	600 ± 24	8.0
	9	3	576	600	529	568 ± 36	12.4
	10	3	650	576	600	608 ± 38	12.4
C	11	3	546	513	483	514 ± 30	12.0
	12	3	528	528	550	536 ± 12	5.0
	13	3	528	528	573	542 ± 24	9.0
	14	3	525	480	480	500 ± 26	10.0
	15	3	546	550	572	556 ± 14	5.0
						Total Average	10.2

Variation of the lysis area induced by dialyzed undiluted normal rabbit urine on fibrin plate.

on arithmetic graph paper against its concentration (CTA unit/ml). The resulting urokinase standard curve is shown in Figure 15.

Plasminogen rich fibrinogen prepared in our laboratory according to the method of Cohn was used to make the fibrin plates. Urokinase concentrations below .05 units per ml produced no clear-cut lysis areas. Concentrations above 80 units per ml produced the same lysis areas on the fibrin plates. Therefore, the sensitivity of the fibrin plates used in this study lay between .05 to 80 units per ml of reference urokinase. Undiluted urine samples which produce no lysis area (activity below .05 unit per ml) are considered as having zero urokinase activity. Urine samples with an urokinase activity above 80 units per ml of standard urokinase had no actual readable values. From this observation, the question arose as to whether one should use the urokinase measurement in the diluted urine samples. Theoretically, this would permit reading in the upper range of values in cases where a particular urine sample exhibits high urokinase activity and fibrin plate insensitivity would ordinarily prevent determination of any value. In Figure 15 the results with urokinase activity measured in undiluted urine and in 1:2 and 1:4 diluted urine of the same urine are illustrated (Figure 14).

Plasminogen determination: Plasminogen in plasma was first converted to plasmin by a plasminogen activator and the resulting proteolytic activity was measured by the procedure of Girolami, et al., 1967. This assay was performed as follows: 0.5 ml of citrated plasma (four parts of blood to one part of 3.8 percent sodium citrate) was first adjusted to pH 2 with HCl and left at room temperature for 15-20 minutes to destroy the antiplasmin. The plasma was then brought back to pH 7.4

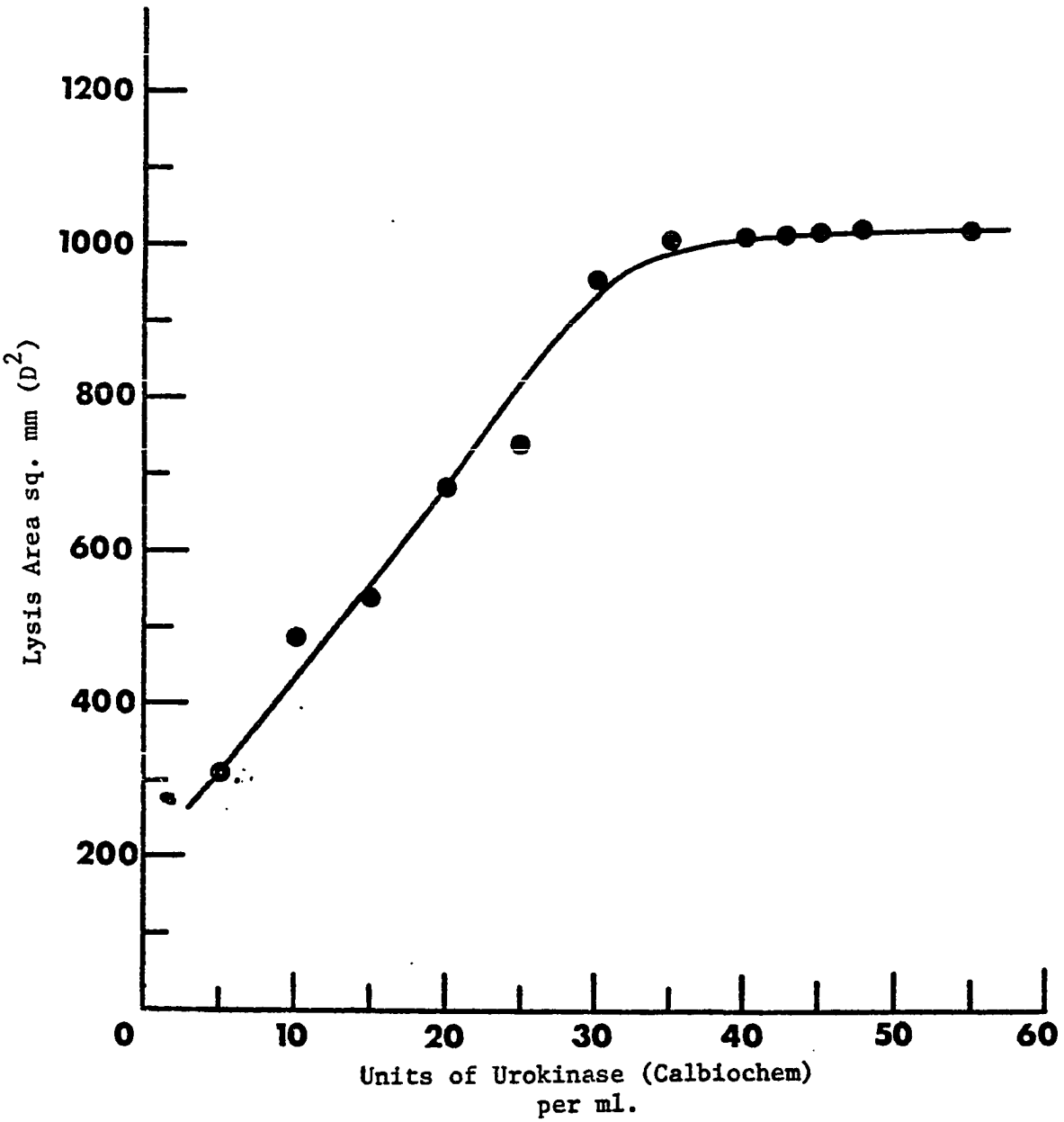


FIGURE 15. Urokinase Standard Curve.
Abscisca: Standard urokinase CTA units per ml.
Ordinate: The lysis area in mm^2 on unheated fibrin plate.

with NaOH and 0.1 phosphate buffer added to make a total volume of one ml. Streptokinase (Varidase) 1000 units or urokinase 100 CTA-units were added to activate the plasma plasminogen; and then 4 ml of 2.5 percent casein (Hammersten, Vitamin-free) in 0.1 M phosphate buffer was immediately pipetted in and mixed well. A 2 ml aliquot was immediately taken and added to 5 ml of a 7 percent thrichloroacetic acid to stop the enzymatic action. This was zero time plasmin activity.

Another 2 ml aliquot was again withdrawn after one hour incubation. The supernatant after cold centrifugation was used to determine the proteolytic activity at 275 millimicron in a Beckman D.B. spectrophotometer. The difference between the readings at zero time and at one hour incubation would represent formed plasmin converted from plasminogen in the test solution. Activity was expressed as gamma of tyrosine was released and was read from the tyrosin standard curve. (Figure 16.)

3.13 Determination of Fibrinogen:

3.13.1 Spectrophotometric method:

Fibrinogen and fibrin were determined spectrophotometrically. The fibrinogen content was calculated from the amount of fibrin formed under standard conditions by addition of bovine thrombin to a fibrinogen solution. For the coagulation and syneresis the method of Morrison, (1947) was used.

The coagulation was performed at pH 6.35±0.05 and ionic strength 0.15. The protein concentration in the coagulation mixture was between 0.5 and 1.5 g/litre. The procedure is as follows; 1.0 ml of the fibrinogen solution (0.15-0.35) in 0.15 M sodium chloride was pipetted off into a small beaker (interior diameter 25 mm) and 2.0 ml of a phosphate buffer

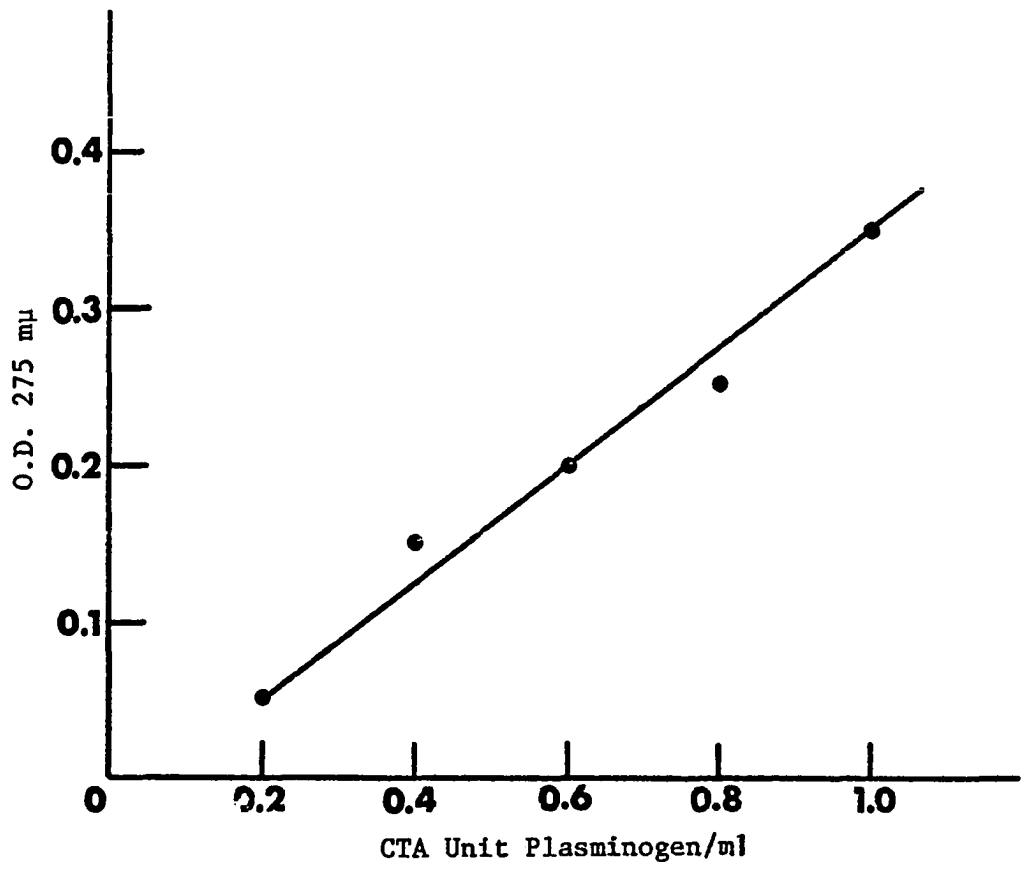


FIGURE 16. Plasminogen (at natural pH) assayed and α-casein used as a secondary substrate in caseinolytic assay.

solution of pH 6.35 and ionic strength 0.15 were added. (The composition of this buffer: 0.005 M $Na_2HPO_4 \cdot 2H_2O$, .010 M KH_2PO_4 and 0.12 M NaCl). 0.15 ml of a thrombin (bovine) solution containing 100 NIH units/ml was then added under mixing. The thrombin contained 100 NIH units/ml and was free of fibrinolytic enzymes.

The coagulation mixture was left standing at room temperature for two hours. The clot was then carefully poured on a small slick cloth (10x10 cm) with several underlying coarse filter papers and allowed to synerize completely. It was then immediately washed three times with 0.15 M sodium chloride, after which the synerized clot was wound up on a glass rod. Drying of the clot had to be avoided, not to render the subsequent dissolution of the clot difficult.

The glass rod with the synerized clot was placed in a test tube with a ground-in stopper. Ten ml of 40% urea in 0.2 N sodium hydroxide were added, and the contents were mixed by whirling. After 60 minutes, when the clot was dissolved, 1.0 ml of 0.15 M sodium chloride was added and the contents were carefully mixed. Within two to four hours after the addition of urea the extinction values were read in a Beckman spectrophotometer, type DB, at 282 mμ in a 1 cm cell against alkaline urea containing the same amount of sodium chloride solution. Slit: about 0.3 mm.

The extinction coefficients ($E^{1\%}_{1cm}$) for bovine fibrin and fibrinogen in alkaline urea are 16.84 and 16.51 respectively. The intercept of the protein dilution curve with the ordinate is 0.01. The following formulas may then be used for the calculation of the fibrin content and the amount of fibrinogen corresponding to the fibrin.

$$\frac{E - 0.01}{0.153} = \text{mg fibrin per ml original sample.}$$

$$\frac{E - 0.01}{0.150} = \text{mg fibrinogen per ml original sample.}$$

For fibrinogen determination in citrated plasma a somewhat modified procedure was used on account of the high buffering capacity of plasma. This is briefly as follows: To 0.6 ml of plasma was added 0.4 ml of 0.15 M sodium chloride and 2.0 ml phosphate buffer, pH 6.0, ionic strength 0.15. The pH value in the mixture will then be between 6.3 and 6.4 and the ionic strength about 0.15. In other respects the procedure was identical with that just described. As regards plasma fibrinogen determinations a comprehensive review of different methods has been given by Jacobsson (1955).

Determination of total protein in the fibrinogen preparations procedure was as follows; 1.0 ml of the fibrinogen solution (0.15-0.35%) in 0.15 M NaCl was pipetted off into a test tube and 10.0 ml of 40% urea in 0.2 N NaOH were added. The extinction was read at 282 mμ within two to four hours after the addition of urea.

When calculating the protein content the extinction coefficient of bovine fibrinogen, 16.51 was used.

Thus,

$$\frac{E - 0.01}{0.150} = \text{mg protein per ml original sample.}$$

Coagulability is the ratio of fibrinogen to total protein in the fibrinogen preparations.

$$\% \text{ clottability} = \frac{\text{Fibrin (mg/ml)}}{\text{Fibrinogen (mg/ml)}} \times 100$$

3.13.2 Assay of Fibrinogen in Plasma:

(1) Blood samples were collected in heparin (dissolved in a minimal volume to avoid unnecessary dilution). The cells were centrifuged and the supernatant plasma was drawn off which was ready to assay.

(2) Plasma samples were diluted. Since the sensitivity of the assay was 2 µg per ml it was necessary to dilute the plasma approximately a thousand-fold. Three suitable dilutions were made for screening plasma fibrinogen levels were 1:500, 1:1000, 1:2000.

(3) One drop of each dilution was transferred to a previously marked circle on the glass test slide.

(4) The latex suspension was mixed by shaking the container vigorously for a few moments and then one drop of latex was added to each plasma dilution on the test slide.

(5) Each of the plasma/latex mixtures was mixed by a disposable mixing rod and each pool of liquid was spreaded to fill the circle.

(6) The slide was racked gently for two minutes before looking for microscopic agglutination.

Readings of Results:

An agglutinated pattern in any position on the glass slide indicated the presence of fibrinogen at a final concentration greater than 2 µg per ml in the plasma dilution in that position. A positive reaction in the 1:500 dilution was indicated a fibrinogen level in the original serum sample at a concentration in excess of 100 mg per 100 ml, in the 1:1000 dilution a level greater than 200 mg per 100 ml and in the 1:2000 dilution a level greater than 400 mg per 100 ml.

3.13.3 Modified Micro-Kjeldahl Method for Fibrinogen Determination:

The method was recommended for the use of carbon-hydrogen, nitrogen, halogen, and sulfur determinations (Steyermark, et al., 1949). The modified Micro method was used for the determination of nitrogen in the clot which is the indirect measurement of the fibrinogen content of the clot. To form the clot, 2 ml of .85% saline, 0.1 ml of 1% calcium chloride and 0.5 ml citrated plasma (4% citrate) placed in a test tube and 10 NIH units of thrombin (Topical, Parke-Davis) added and gently shook to mix the reaction mixture well. The mixture was incubated in 37°C water bath for 30 minutes. After 30 minutes of incubation the fibrinogen polymerized and fibrin clot formed.

The clot was winded with a glass rod and bolted on filter paper to take the moisture of the clot. The winded clot was put in Kjeldahl flask and washed with 0.85% saline twice in 30 minutes and decanded the wash solution (saline). To the clot 2 ml of digestion mixture was added and allowed the clot to digest in the digestion mixture for 24 hours. After 24 hours of digestion, the digested clot was placed on the flame in order to cook for 8 hours. After the cooking, 0.5 ml of superoxal was added and cooked additional 30 minutes.

Distillation: Digested clot was removed from the fire and cooled before distillation. In order to run a blank, 2.0 ml of digestion mixture was added to the funnel and rinsed with small amounts of triple distilled water. The stopcock was closed after the formation of bubbles in the reservoir. The 5 ml of Boric acid was placed in 50 ml of Erhlenmeyer flask and put on the end of the outlet tube. The gas was allowed

to bubble through indicator until the flask was 2/3 full. The flask was removed and titrated with standardized acid (N/100 HCl). Reading-blank-X x Factor of acid-fibrinogen in mg%. In the Kjeldahl distillation apparatus (Figure 17) changes were made in the conventional designs of the distillation flask and the condenser tube had to be lubricated with stopcock grease and held together with a suitable clamp, and a west-type condenser (Scientific Glass Apparatus Co., Inc., Bloomfield, N.J.).

Operation: With both stopcocks open, the sample was introduced through the funnel and the curved tube into the inner chamber or distillation flask. The alkali (saturated NaOH) was added in like manner, displacing the acid portion upward. Steam was generated with the stopcocks on both the sample inlet funnel and the drainage tube closed and the distillation was allowed to proceed.

Several methods of cleaning could be used. The following one had been found satisfactory, particularly when boiling chips were employed in the digestion: While steam was being generated and with both stopcocks closed, the sample inlet funnel was filled with water and the tip of the condenser immersed in about 100 ml. of distilled water contained in a beaker. Steam generation was stopped, and the stopcock of the funnel was opened slowly, being closed before all the water had drained into the apparatus. Reduced pressure caused the water to be sucked from the beaker into the apparatus, washing it. The stopcock in the drainage tube was opened to empty the liquid which had collected in the outer jacket, the steam generation was started again, and the procedure was repeated.

The inner portion of the Kjeldahl apparatus was heated by steam flowing through the system, in contrast to maintenance of the temperature

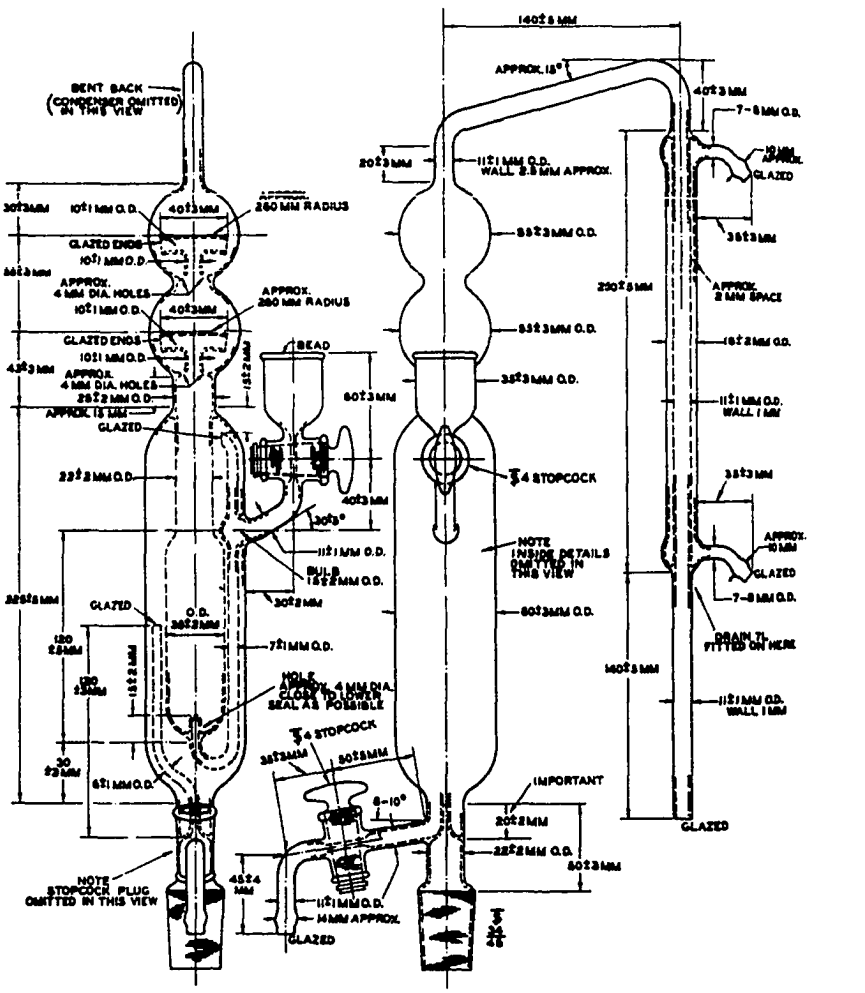


FIGURE 17. Kjeldahl Distillation Apparatus: Changes have been made on a conventional design of the distillation flask, and the condenser tube, they include a ball and socket joint connection.

by a vacuum jacket. The steam entered through the vertical tube extending almost halfway up the outer jacket at the left, surrounded the distillation flask proper (inner jacket), and passed into the small bent tube near the top right (above the sample tunnel inlet), and then downward through the bent portion of the tube at the lower end of the inner jacket and up into the center portion of the distillation flask. The two traps with T shaped tubes held back alkali spray.

Reagents for Kjeldahl method:

(1) Digestion Mixture:

600 ml. sulfuric acid (H_2SO_4)

200 ml. phosphoric acid (H_3PO_4)

8 gm. Cupric sulfate ($CuSO_4$)

8 gm. Selenium dioxide (SeO_2)

Acids were mixed and the mixture was divided into two separate containers. The $CuSO_4$ was dissolved in one part and the SeO_2 in the other, let the solution settle for 24 hours then recombined.

(2) Boric Acid indicator (stored in dark bottle)

100 gm. Boric Acid

20 ml. 0.1% Bromcesol green in 95% ethanol

10 ml. 0.1% methyl red solution in 95% ethanol

Diluted with distilled water to 2000 ml. (this solution was supersaturated).

(3) Saturated NaOH.

(4) Standardized N/100 HCl.

(5) Superoxal (30% H_2O_2: Merck and Co.)

Results:

Fibrinogen values in the test population ranged 155 to 220 mg percent with a mean of 194 mg%. Fibrinogen values in the population (control ranged from 300 to 410 mg percent with the mean of 330 mg%. (Figure 18.)

The t-tests were used as a test for significance between the rabbits eight week mean values of both the test and control populations. The results of these tests were highly significant. By establishing the level of significance to be accepted at 0.05 or 95 percent the t-test of the eight weeks mean values of both the exposed and the control populations as groups gave a level of significance of $P > 0.001$, a highly significant difference.

3.14 Rapid Sensitive Method for Measuring Fibrin/Fibrinogen Split Products in Rabbit Serum:

The technique of Merskey and co-workers has been modified by using the reagents and method of reading hemagglutination patterns devised by Wegmann and Smithies (Wegmann, et al., 1966). A method has emerged which can be completed within an hour after receipt of the serum sample. It is sensitive enough to detect minute amounts of fibrin split-products in 95% of normal serum.

Principle: Serially diluted samples of serum to be tested were incubated with antifibrogen rabbit serum. If there were no fibrinogen split-products in the diluted test serum, the mixtures retain a full amount of fibrinogen antibody; the more split-products present in serum the less residual antibody was found in the incubation mixture. After incubation the mixtures were added to a suspension of tanned, formalinized,

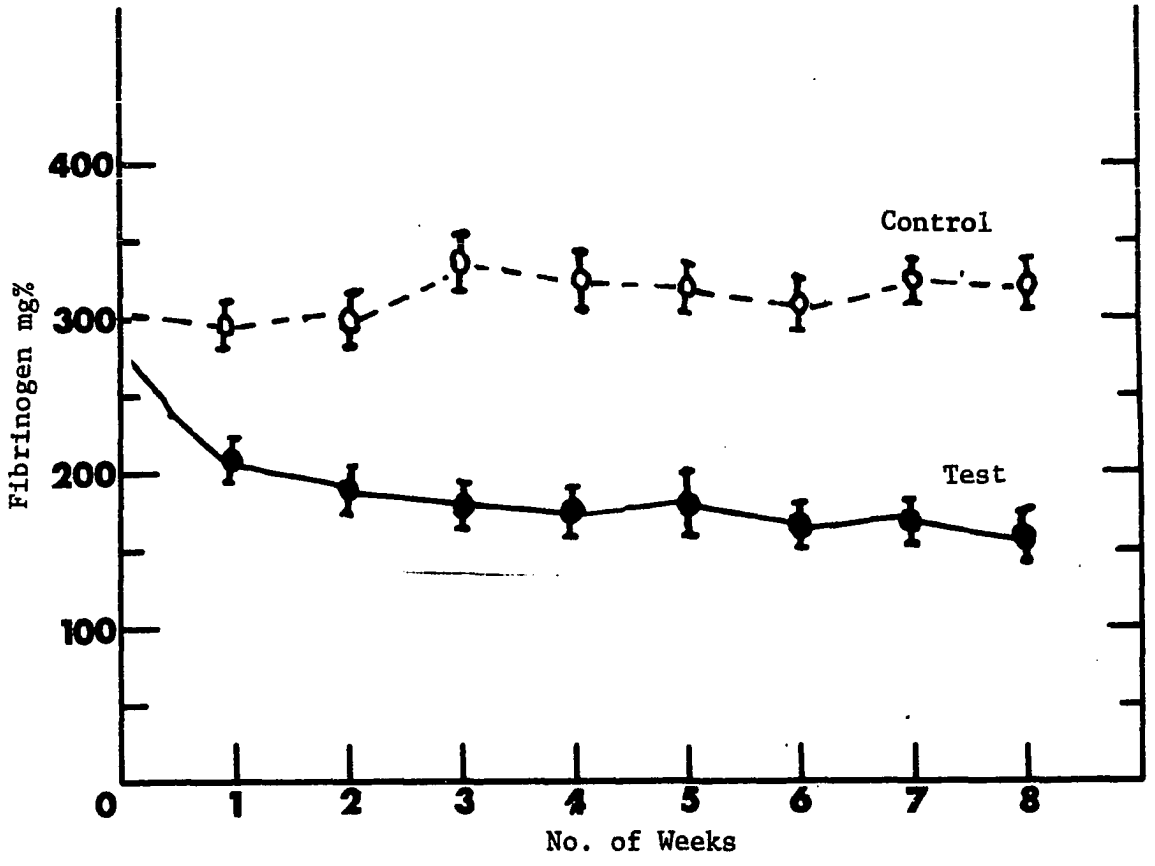


FIGURE 18. Fibrinogen concentrations in the plasma of exposed rabbits are reduced due to the partial degradation of fibrinogen molecule.

fibrinogen-coated, erythrocytes. The amount of split-products could be estimated from the dilution of serum which inhibited the agglutination of the coated cells. The more residual antibody in serum mixtures the lower the titer of inhibition of erythrocyte agglutination and, thus, the higher the titer of inhibition the higher the concentration of split-products in the test serum.

3.14.1 Preparation of Fibrinogen Coated Erythrocytes:

(1) Nine volumes of group O human blood were mixed with one volume of 0.1 M sodium oxalate.

(2) The erythrocytes were separated by centrifugation and washed four times with 0.9% sodium chloride solution.

(3) Formalinization: To 1 volume of 3% formaldehyde in saline (previously brought to pH 7.2 to 7.4 with sodium hydroxide). The cells were kept suspended with a magnetic stirer (slow speed) for 20 to 24 hours at 37°C and then were washed three times with saline and stored as a 20% suspension at 5°C in phosphate-citrate buffer (equal volumes of 0.15 M phosphate buffer, pH 6.4, and 0.1 M sodium citrate containing 0.1% sodium azide). Such cells were stable for at least several months.

(4) Tanning: The 20% suspension of formalinized erythrocytes is diluted 10-fold with phosphate-citrate buffer. Equal volumes of this 2% cell suspension and 1:40,000 freshly prepared tannic acid in the same buffer were incubated for 1 hour at 56°C with gentle mixing every 20 minutes. The tanned cells were washed three times with phosphate-citrate buffer.

(5) Fibrinogen coating of erythrocytes: A 4% suspension of formalinized, tanned erythrocytes in phosphate-citrate buffer was mixed with

an equal volume of fresh, oxalated, normal, plasma which had been diluted 250-fold in the same buffer. The mixture was incubated for 1 hour at 37°C with gentle mixing. The cells were washed three times in phosphate-citrate buffer and suspended, at 10% concentration, in a phosphate-citrate-albumin buffer (0.4 bovin serum albumin in the phosphate-citrate buffer). When stored at 5°C, this stock suspension was stable for at least 2 months. One milliliter of packed cells was sufficient for several hundred assays.

Test Serum: The rabbit's freshly drawn blood was mixed with soybean trypsin inhibitor (2 mg/2 ml of blood) (type I-S, Sigma Chemical Company) to prevent fibrinogenolysis after collection. The mixture was promptly inverted three times and then was allowed to clot at room temperature until retraction was evident. The serum was harvested after centrifugation and was stored, if need be, at 5°C (at which temperature was stable for several days) or at -20°C (at which the fibrinogen split-products remained unaltered indefinitely).

Antifibrinogen Rabbit Serum: This was obtained from Hyland (Los Angeles) and was absorbed with 1/4 volume of erythrocytes at 5°C overnight. The antifibrinogen serum was first serially diluted to determine the highest dilution which will strongly agglutinate erythrocytes coated with fibrinogen. The technique described in the following paragraph was used. Antiserum was substituted for the rabbit's serum and then coated cells were added. The highest dilution of the antiserum yielding 4+ agglutination was 1:40,000. This antiserum was used in the hemagglutination-inhibition assay at one dilution less (1:20,000) than the highest dilution.

Assay Procedure: (1) The test serum was serially diluted. To

every well of a type U microtiter plate was added 0.025 ml of phosphate-citrate-albumin buffer with a pipet dropper. Then, 0.025 ml of test serum was placed in the first well of each row (duplicates) with microdiluters. The serum and buffer were mixed by swirling the microdiluters 20 times. The diluters were then transferred to the next wells of the rows, carrying along 0.025 ml of the mixture. Simultaneous dilutions of as many as eight rows could be carried out by placing the stems of the microdiluters against a glass rod covered by rubber tubing, to keep them evenly lined up during the transfer (Figure 19).

(2) Antifibrinogen serum diluted 1:20,000 was added (0.025 ml to each well) with a pipet dropper.

(3) The plate was vibrated for 60 seconds and then incubated at 30°C for 10 minutes.

(4) The formalinized, tanned, fibrinogen-coated erythrocytes in 1:1250 suspension in phosphate-citrate-albumin buffer were added (0.025 ml to each well) by a pipet dropper.

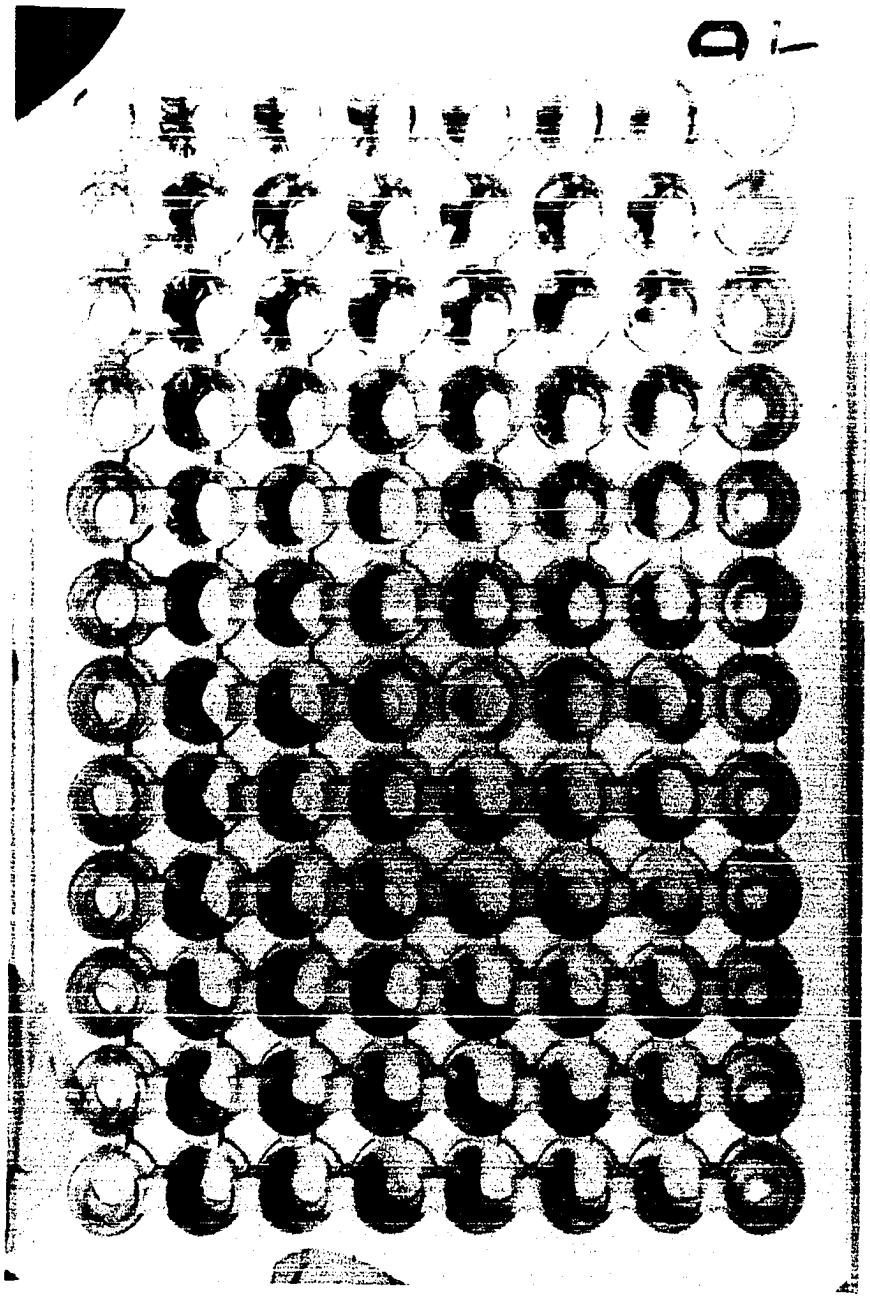
(5) The plate was again incubated for 10 minutes at 30°C.

(6) The plate was centrifuged at 1,500 rpm for 30 seconds. It was then placed almost vertically (80°) against a white-light viewbox.

(7) The button of cells in each well was observed closely for 5 to 15 minutes for the developing pattern and was graded as 0 to 4+. A negative pattern (inhibition of agglutination) was represented by early migration of the cell button into a narrow line or tail. Grade 4+ agglutination was represented by absence of tailing and persistence of agglutinated cell button in its original rounded form. Grades 1+, 2+, and 3+ represented intermediate stages between prominent tailing with a small

FIGURE 19. Typical result with microtitre plate from the inspection of the standard titration (row B) the sensitivity of the assay determined. (That is concentration of fibrinogen at the end point.)

The end-points of the samples were read in turn. Dilution factor at the end-point multiplied by the sensitivity of the assay to give the concentration of fibrinogen or its degradation products in the undiluted sample.

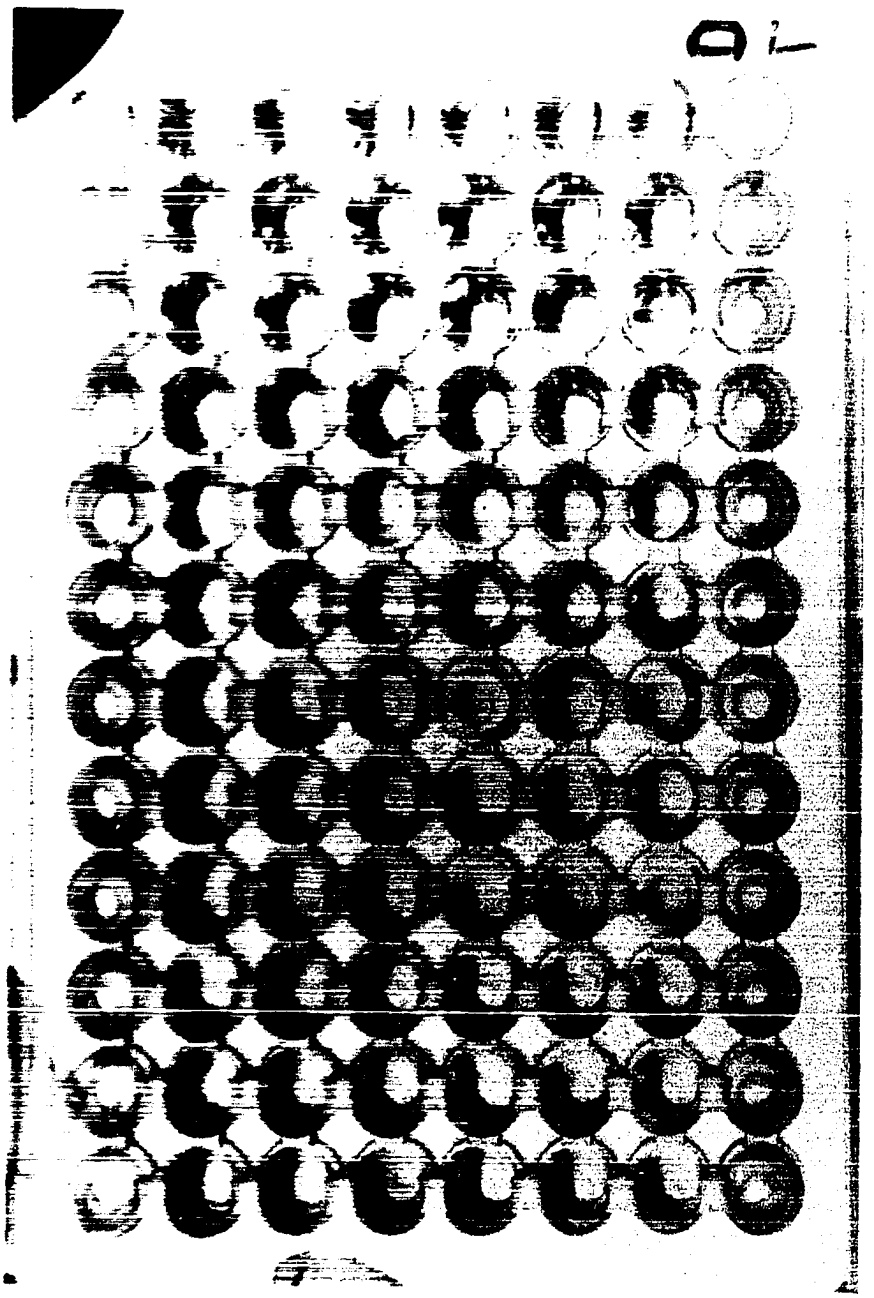


residual button and only little tailing with a prominent button. The titer of inhibition was judged to be the highest dilution of test serum yielding less than a 3+ pattern.

(8) Fibrinogen control: The fibrinogen concentration in a sample of normal plasma was evaluated by thrombin clottability (Morrison, 1947). The plasma was then diluted in phosphate-citrate-albumin buffer to a fibrinogen concentration of 1 mg/100 ml (usually about 300-fold) and stored at -60°C. The diluted plasma was titrated simultaneously with every plate, exactly at the test serums.

(9) Nonspecific hemagglutination: Occasionally, the control well containing test serum, buffer, and coated cells (but no antifibrinogen serum) will show agglutination, probably because of antibody in the test serum against formalinized cells. This nonspecific hemagglutination can be eliminated by absorbing 2 vol. of the test serum with 1 vol. of packed, formalinized, group O, human erythrocytes for 10 minutes at 5°C.

Calculation and Presentation of Results: Although the split-products of fibrinogen in serum were actually being detected, the value was expressed in terms of the whole fibrinogen standard in the control plasma. If the 1 mg/100 ml solution of fibrinogen (plasma) reaches an agglutination-inhibition end point at a dilution of 1:8, the concentration of fibrinogen in the plasma in the final well was 0.125 mg/100 ml or, more conveniently, 1.25 µg/ml. Any serum is then said to have fibrinogen split-products equivalent to 1.25 µg of fibrinogen per milliliter at its own end point. The concentration of split-products is thus the reciprocal dilution of the serum multiplied by 1.25 and was expressed as µg/ml.



residual button and only little tailing with a prominent button. The titer of inhibition was judged to be the highest dilution of test serum yielding less than a 3+ pattern.

(8) Fibrinogen control: The fibrinogen concentration in a sample of normal plasma was evaluated by thrombin clottability (Morrison, 1947). The plasma was then diluted in phosphate-citrate-albumin buffer to a fibrinogen concentration of 1 mg/100 ml (usually about 300-fold) and stored at -60°C. The diluted plasma was titrated simultaneously with every plate, exactly at the test serums.

(9) Nonsp[illegible]tination: Occasionally, the control well containing t[illegible]ated cells (but no antifibrinogen serum) wil[illegible]y because of antibody in the test serum aga[illegible] nonspecific hemagglutination can be elimina[illegible]e test serum with 1 vol. of packed, form[illegible]erythrocytes for 10 minutes at 5°C.

Calcula[illegible]on of Results: Although the split-products of fibrinogen in serum were actually being detected, the value was expressed in terms of the whole fibrinogen standard in the control plasma. If the 1 mg/100 ml solution of fibrinogen (plasma) reaches an agglutination-inhibition end point at a dilution of 1:8, the concentration of fibrinogen in the plasma in the final well was 0.125 mg/100 ml or, more conveniently, 1.25 µg/ml. Any serum is then said to have fibrinogen split-products equivalent to 1.25 µg of fibrinogen per milliliter at its own end point. The concentration of split-products is thus the reciprocal dilution of the serum multiplied by 1.25 and was expressed as µg/ml.

Fibrinogen Split-products in test serum (FSP)	X	Inhibition titer of test serum	=	Fibrinogen in control plasma	X	Inhibition titer of control plasma

or

$$\text{Fibrin split-products (FSP) in test serum} = \frac{\text{Fibrinogen in control plasma} \times \text{Inhibition titer of control plasma}}{\text{Inhibition titer of test serum}}$$

Results: The results were summarized in Figure which compiles the effect of CO on fibrinolytic activity. Increased fibrinolytic activity was induced in the animals that were exposed to CO within the wide range from 20 µg/ml to 80 µg/ml of fibrin degradation products. From the Figure [20] it is clear that the fibrinolytic enzyme system was not exhausted after certain time period. These observations would possibly indicate that blood of exposed rabbits contains an amount of plasminogen activator sufficient to get up to 80 µg/ml fibrin/fibrinogen degradation products in serum.

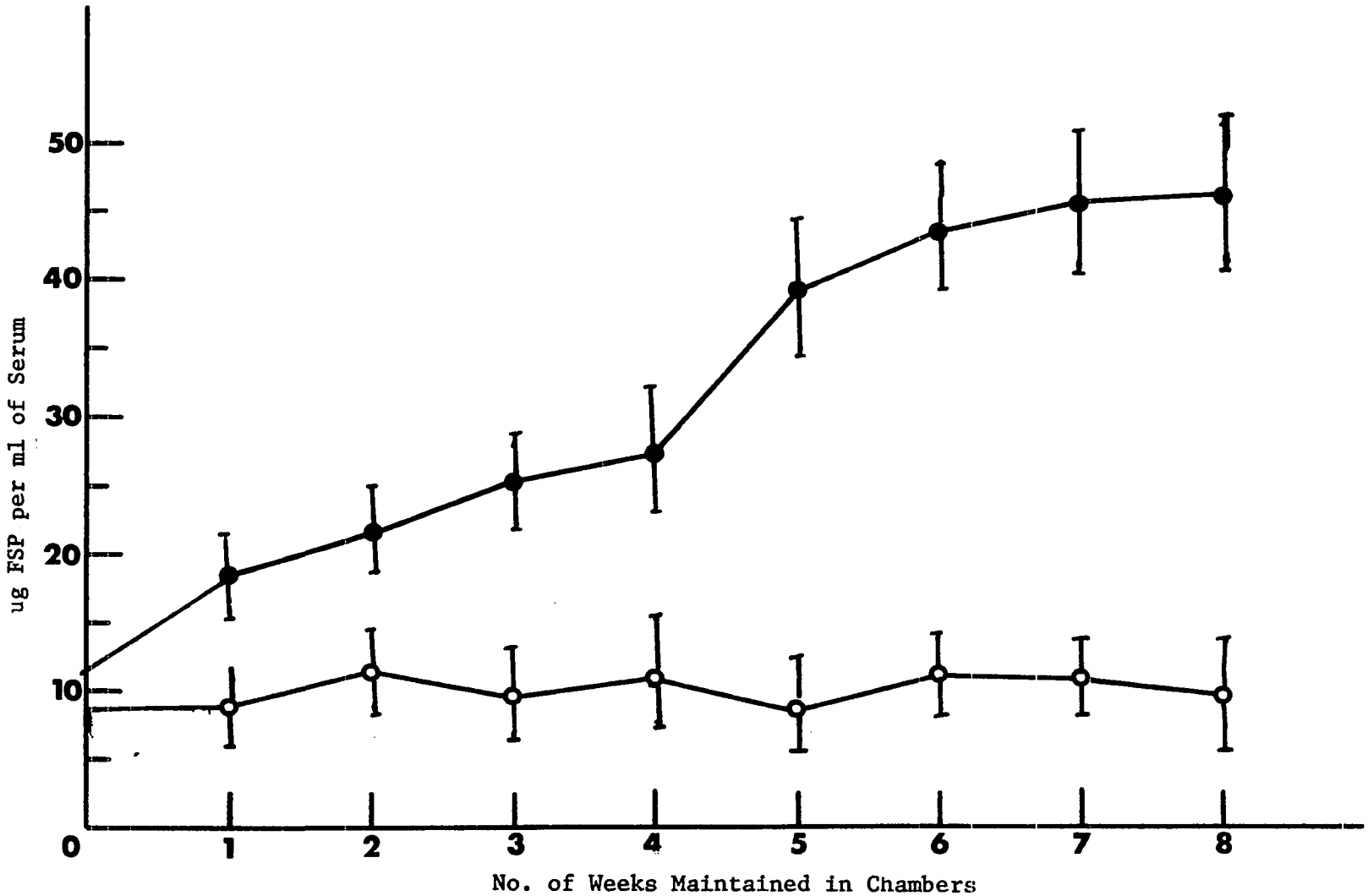
3.15 Immunodiffusion Plate Technique for α_2-Macroglobulin Test:

Serum proteins are of such a complex chemical nature that they have been difficult to identify or to differentiate by conventional chemical and electrophoretic techniques. By usual electrophoretic methods, serum proteins are classified into groups designated as albumin, $Alpha_1$ globulins, $alpha_2$ globulins, beta globulins, and gamma globulins. A number of protein fractions present in serum are not readily distinguishable by electrophoresis, primarily because of similar or overlapping electrophoretic mobilities. Immunodiffusion techniques are specific for identification and quantitation of those proteins for which specific antibodies have been developed.

FIGURE 20. The concentration of split-products in the serum of exposed (test) rabbits increased due to the degradation of fibrinogen and/or fibrin.

●–● Test

○–○ Control


50
40
30
20
10
0
1
2
3
4
5
6
7
8
ug FSP per ml of Serum
No. of Weeks Maintained in Chambers

In the immunodiffusion plate the antigen placed in a well in antibody-containing agar gel diffused into the agar and formed a precipitin ring. (See Figure 21) The diameter of this ring is directly related to the concentration of the antigen. Each immunodiffusion plate contained six wells, three wells for the three reference serums which were provided with the test kit. Thus, one plate was used to prepare a reference curve and simultaneously to test three unknown serum samples. Performance of the test and the determination of concentration of unknown sample were the same as explained before.

Results: The formation of precipitin rings were poor. No significant difference of the precipitin rings between exposed and control group was found. There was no significant difference in the diameter of the precipitin rings and those with reduced but measurable areas gave the p-value > .50. Each sample was tested on three plates and applied in duplicate on each plate. Some variation in measurement was found between samples. The precipitin ring areas were calculated by DxD.

3.16 Quantitative alpha-1-antitrypsin test:

Alpha-1-Antitrypsin (α,AT), the major protease inhibitor of plasma (Briscoe, et al., 1966a; Miesch, et al., 1971b) is a polymorphic system of proteins which may be identified in a series of zones between orosomucoid and albumin when plasma is subjected to discontinuous starch gel electrophoresis at pH 4.5 (Kueppers, et al., 1966a; Fagerhol, et al., 1967b). The abbreviation Pi (protease inhibitor) has been suggested for this complex protein system, and seven co-dominant alleles were proposed to genetically explain the system (Fagerhol, et al., 1967). Since α_1AT is only one of several serum protease inhibitors, all of which may vary widely

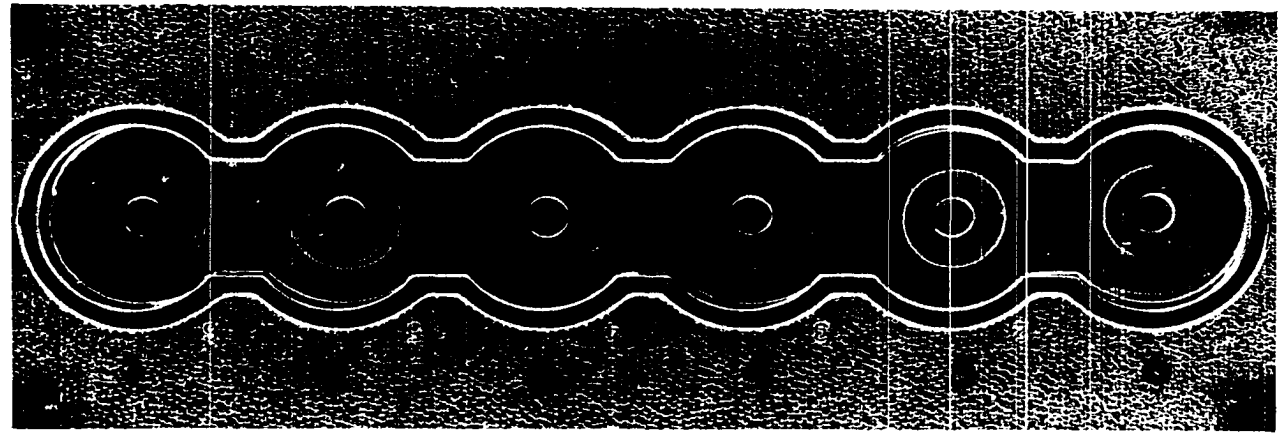


FIGURE 21 The amount of immune precipitate formed is dependent upon the quantity of α_1 AT present in the test sample. There is no significant difference between the test and the control groups. From 1 to 3 control (non-exposed). From 4 to 6 Exposed, test group's samples applied.

in concentration, immunological assays are more specific than enzymatic assays (Tarkoff, et al., 1968).

The serum α_1AT was determined by single radial immunodiffusion. Monospecific antiserum is incorporated within a buffered agar medium in plate and wells of exacting dimension were removed to receive the test sample. The buffered agar used in the plate media in order to allow free diffusion of serum protein while the monospecific antiserum restricts the diffusion of the α_1AT by immune precipitation. The amount of immune precipitate formed is dependent upon the quantity of α_1AT present in the test sample. The antiserum concentration is carefully controlled so that the size of the precipitin ring formed bears a linear relationship to the concentration of the specific protein after incubation for 24 hours at room temperature. The reference curve is prepared by plotting the ring diameters of the three reference sera on semi-logarithmic paper. The concentration of α_1AT is determined by comparing the ring diameter of the test sample to the reference curve. (Figure 22)

Results: Statistical analysis did not reveal any significant differences between the exposed and the control rabbit serums. Figure gives the comparative range of the size of the precipitin rings between exposed group and the control. There was no significant change in the size of the ring (p value of > 0.25).

3.17 Histological Studies: Differentiation of various tissue components:

Staining procedure:

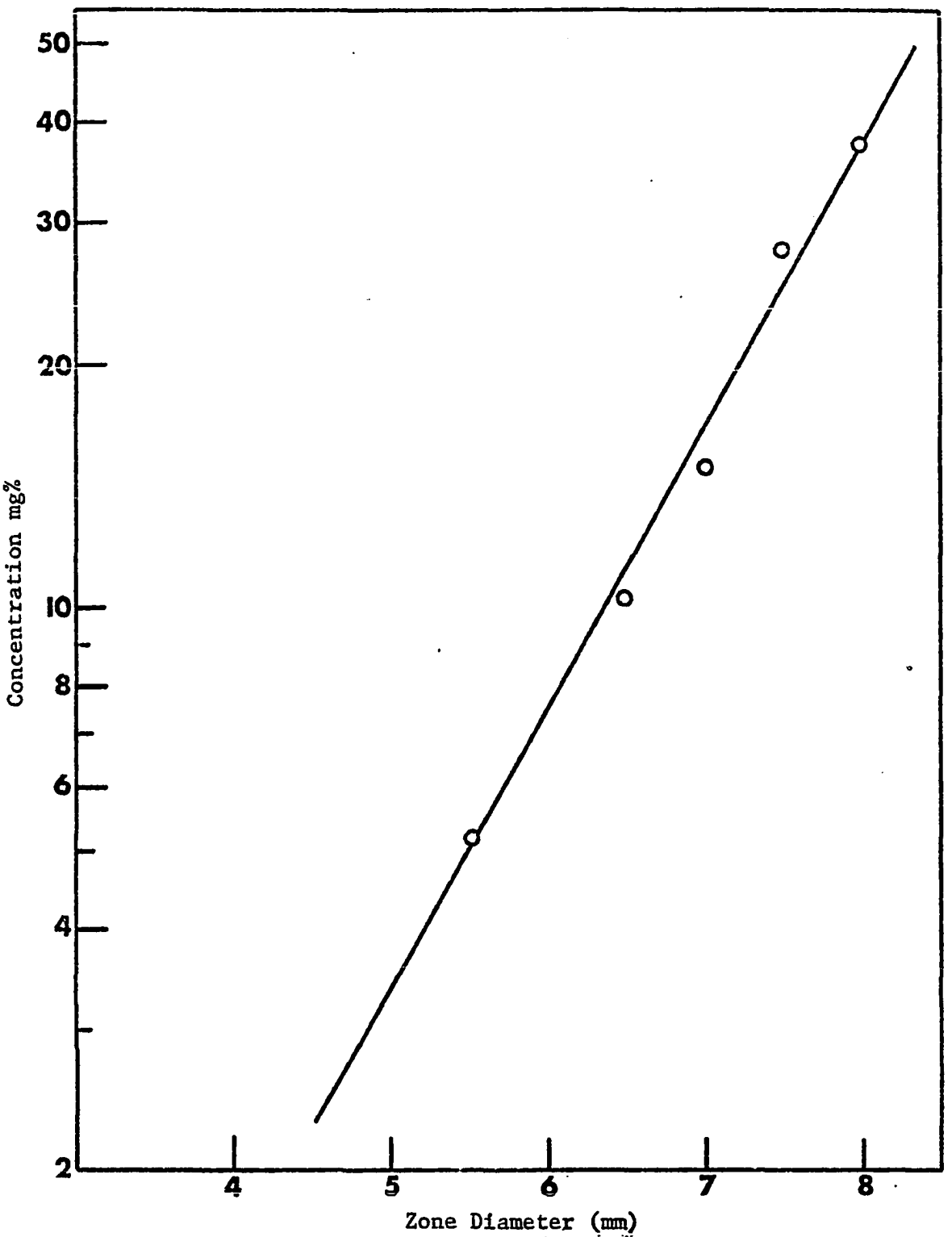
(1) Deparaffinized and hydrated with water.

(2) The mercuric chloride crystals removed with iodine and

FIGURE 22. Immuno-Plate (Reference rabbit serum curve) for α_1 antitrypsin.

a. Horizontal scale, the precipitin ring diameters of three reference serums were plotted.

b. The concentrations of the corresponding reference serums were plotted on the vertical (logarithmic) scale.


50
40
30
20
10
8
6
4
2
Concentration mg%
4
5
6
7
8
Zone Diameter (mm)

cleaned with sodium thiosulphate.

(3) The slides were immersed in Harris' hematoxylin for 15 minutes then rinsed with tap water.

(4) Differentiated in acid alcohol with five to eight quick dips. The differentiation was checked by microscope.

(5) Washed in tap water very briefly.

(6) Dipped in ammonia water or lithium carbonate water until the sections were bright blue.

(7) Washed in tap water for 15 minutes.

(8) Stained with eosin for 15 seconds to 2 minutes.

(9) Dehydrated in 95% and absolute alcohol until excess eosin was removed (in order to be sure checked under microscope).

(10) Absolute alcohol, two changes for 3 minutes each.

(11) Xylene, two changes of 2 minutes each.

(12) Mount in permount of Histoclad.

3.17.1 Azocarmine G Solution

Azocarmine G	1 to 1.5 gm.
Distilled water	200.0 ml

The reaction mixture was brought to a boil and filtered through coarse filter paper in paraffin oven at 50°C, so that the fine particles of dye would pass through. When the mixture cooled 2 ml of glacial acetic acid was added and refrigerated. It was filtered before use.

3.17.2 Aniline-Alcohol Solution

Aniline oil	1 ml.
Alcohol, 95%, ethyl	100 ml.

1% Glacial Acetic Alcohol

Glacial acetic acid	1 ml.
Alcohol, 95%, ethyl	100 ml.

5% Phosphotungstic Acid Solution

Phosphotungstic acid	5 gm.
Distilled water	100 ml.

Stock Aniline Blue Solution

Aniline blue, water soluble	0.5 gm.
Orange G	2.0 gm.
Distilled water	100.0 ml.
Glacial acetic acid	8.0 ml.

Working Aniline Blue Solution

Aniline blue stock solution	1 part
Distilled water	2 parts

Staining Procedure:

(1) Deparaffinize sections by passing through xylene, absolute and 95% alcohols down to water and removed the mercuric chlorides with iodine.

(2) Rinsed with distilled water.

(3) Stained in azocarmine G solution in a covered dish in the paraffin oven at 58°C for 15 to 20 minutes. Allowed to cool for 5 minutes at room temperature.

(4) Rinsed in distilled water.

(5) Differentiated in the aniline-alcohol solution until cytoplasm and connective tissue were pale pink and nuclei stood out sharply. Control differentiation by rinsing slide in 1%

glacial acetic alcohol and checked with microscope.

(6) Mordant in 5% phosphotungstic acid solution until the connective tissue was completely decolorized 15 minutes to 1 hour. Checked with microscope at 15-minute intervals.

(7) Rinsed quickly in distilled water.

(8) Counterstained for 5 to 30 minutes in the working aniline blue solution until the finest connective tissue fibers were sharply stained.

(9) Examined from time to time with microscope.

(10) Rinsed in distilled water.

(11) Dehydrated quickly through 95% alcohol, two changes of absolute alcohol.

(12) Cleared with two or three changes of xylene, and mounted in permount.*

Results:

Microscopic findings in myocardial tissues were as follows: the capillaries are extended filled with red blood cells which reveal clumping. In some areas, there are slight extra-capillary accumulation of the red blood cells. In other areas, the arterial seems to have a thickened wall, and initiation of infraction is seen, in the myocardial tissues of exposed rabbits. Hardening of arteries and deposits of fatty material, chiefly cholesterol within the inner walls of arteries were seen (Figure 23a,b,c).

3.18 Pilot Study:

In order to make sure that the elevated fibrinolytic activity

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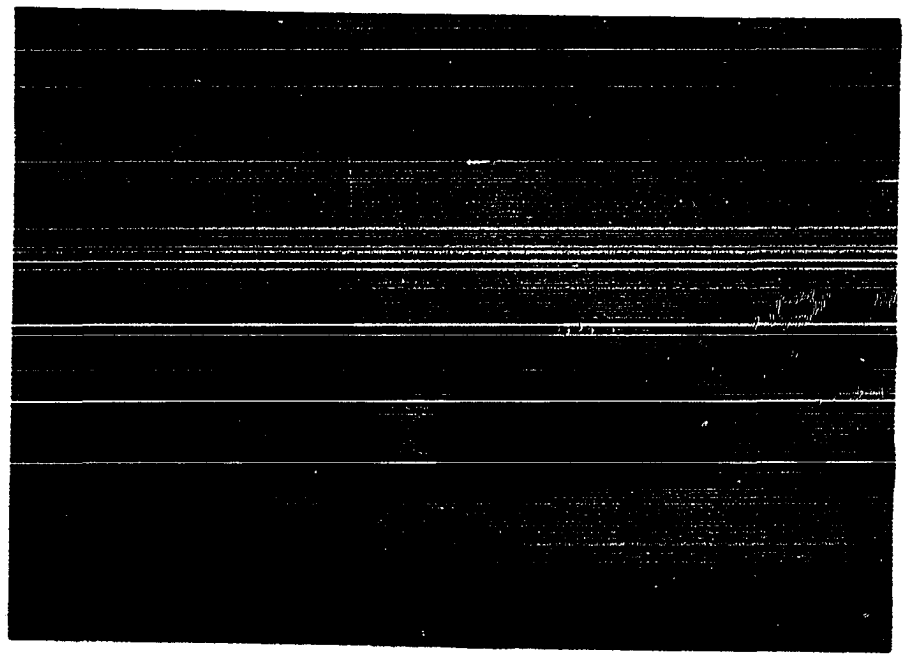
*Pathological Technique Philadelphia: Mallory, F. B., p. 154 (1938).

FIGURE 23. Microscopic studies show major changes in myocardial tissues and arteries (vessel walls).

a) Changes in myocardial tissues
b) Hardening of arteries
c) Thickened vessel walls



was due to the plasminogen activator that was released from the endothelial cells of the vessel walls, a third group of 6 rabbits were maintained under the same environmental condition in chambers. This time, concentration of the CO was increased to 300 ppm/8 hrs. for 5 days a week. A group of 3 rabbits were placed in one chamber and another group of 3 rabbits were placed in the other chamber. Both groups were then given the same concentration of CO (300 ppm/8 hrs) for 4 weeks (Figure 24). Concurrently a group of three rabbits were given epsilon-aminocaproic acid, (EACA, 200 mg/Kg) a known inhibitor of fibrinolysis (through inhibition of the activation of plasminogen). The clots from these animals were visibly different (Figure 25) and fibrin plates showed no lysis (Figure 26) when compared to the blood of the three rabbits receiving 300 ppm CO without EACA. These findings clearly demonstrated that the enhanced fibrinolytic activity was due to the damage of the endothelial cells resulting in a leakage of the "tissue" activator into the circulation.

Blood samples were drawn as described before twice a week for hemoglobin, carboxyhemoglobin, oxyhemoglobin and blood fibrinolytic activity which was measured by whole blood clot lysis, a modified Freanley technique, euglobulin lysis, fibrin plate, caseinolytic assay, fibrinolysis serum fibrin/fibrinogen degradation products. All test results from this pilot study are shown in Figure 27. After two weeks of exposure, the blood fibrinolytic activity was enhanced in the group of 3 rabbits receiving 300 ppm CO/8 hrs for 5 days per week without EACA.

The group of 3 rabbits that received (EACA, 200 mg/Kg with 300 ppm/8 hrs for 5 days per week did not show any fibrinolytic activity in

FIGURE 24. Comparative distribution patterns of O_2, Hb, and COHb in rabbits that were receiving 300 ppm CO 8 hr/day for four weeks (3 rabbits with EACA, 3 without EACA).

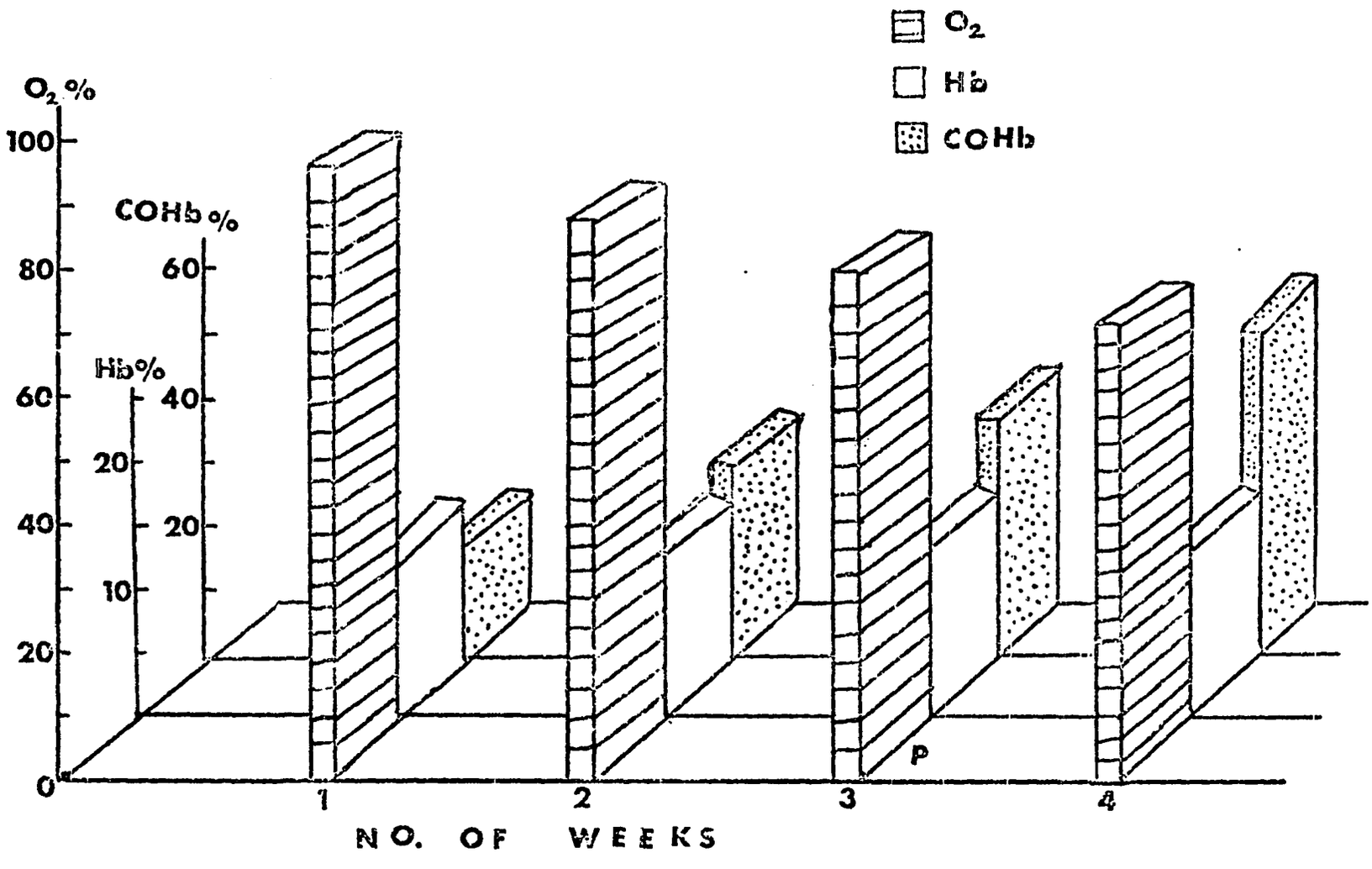

O2
Hb
COHb
O2 %
100
80
60
40
20
0
COHb %
60
40
20
Hb %
20
10
1
2
3
4
P
NO. OF WEEKS

FIGURE 25. Whole blood lysis time: The whole blood from rabbits that were exposed to 300 ppm CO with ε-aminocaproic acid did not show any sign of lysis after one hour of incubation at 37°C water bath (test tubes from 1 to 3). The whole blood clot of rabbits that were exposed to 300 ppm CO without EACA, started to lyse after 20 min. of incubation at 37°C water bath.

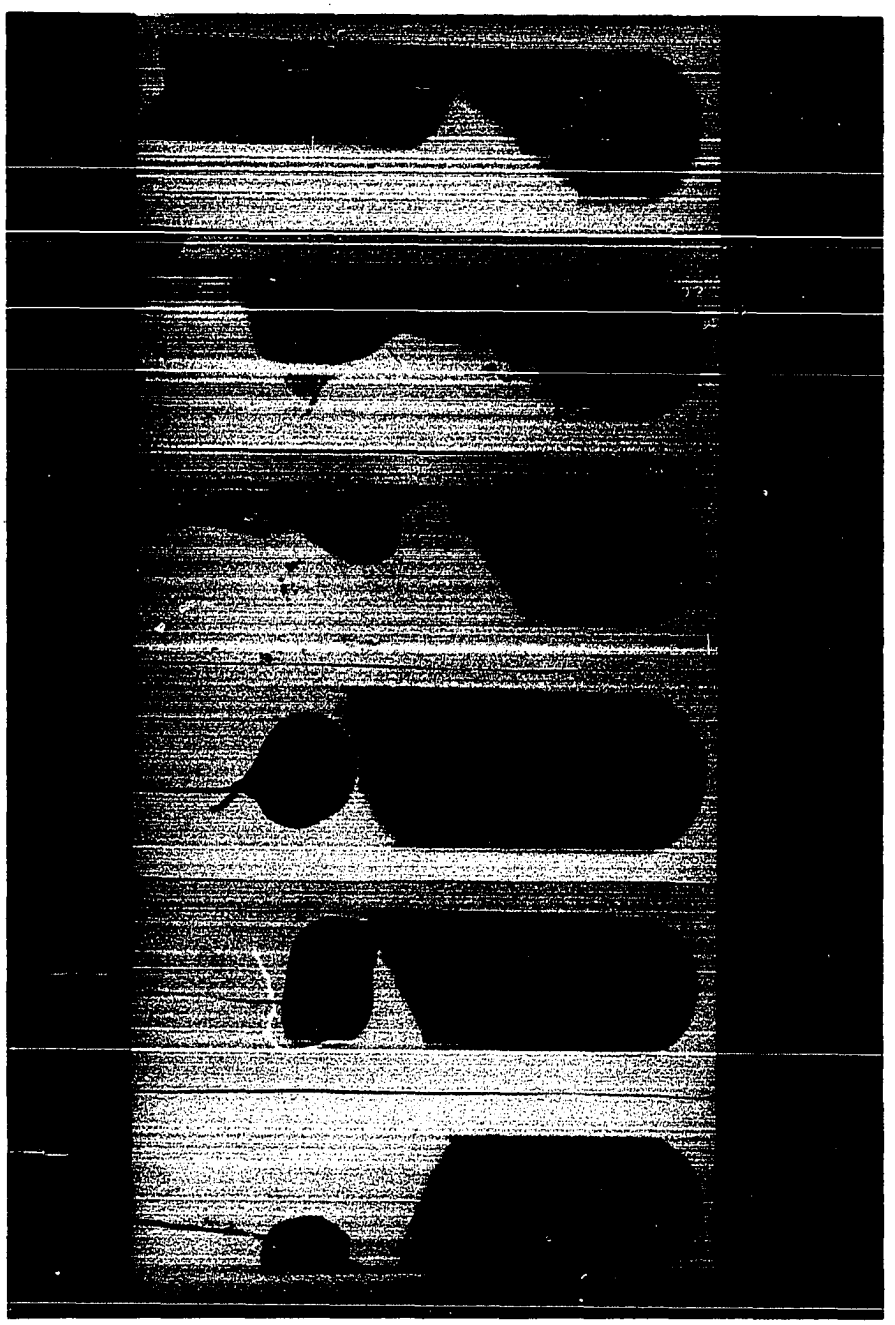


FIGURE 26. Fibrin Plate (Hyland): Plasma samples of rabbits that were exposed to 300 ppm CO with EACA (200 mg/kg) did not show any clear lysis zone on the fibrin plate after incubation at 37°C overnight (wells from 1 to 3). The plasma samples of rabbits that were exposed to 300 ppm CO without EACA (200 mg/kg) did show clear lysis zone areas on fibrin plate (wells from 4 to 6).

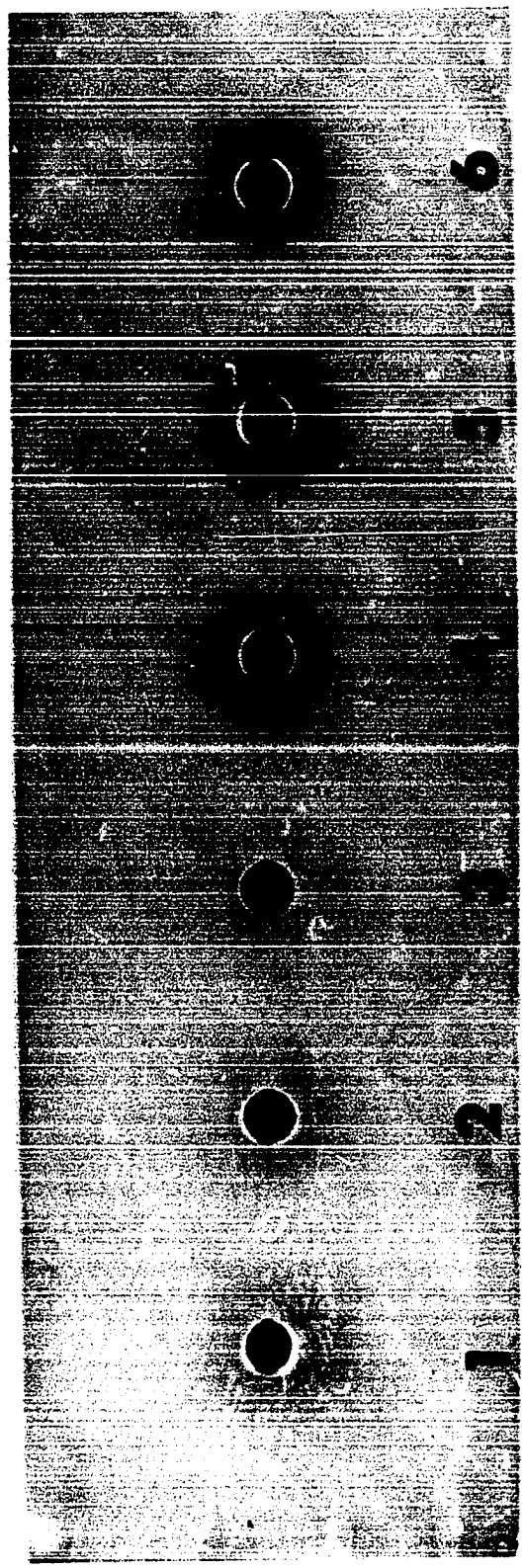

6
5
4
3
2
1

FIGURE 27. Shows changes in major parameters of blood of exposed rabbit (300 ppm CO without EACA). The rabbits exposed to 300 ppm CO without EACA show increase in fibrinolytic activity in all parameters. The rabbits that exposed to 300 ppm CO with EACA do not show elevation of fibrinolytic activity in all parameters.

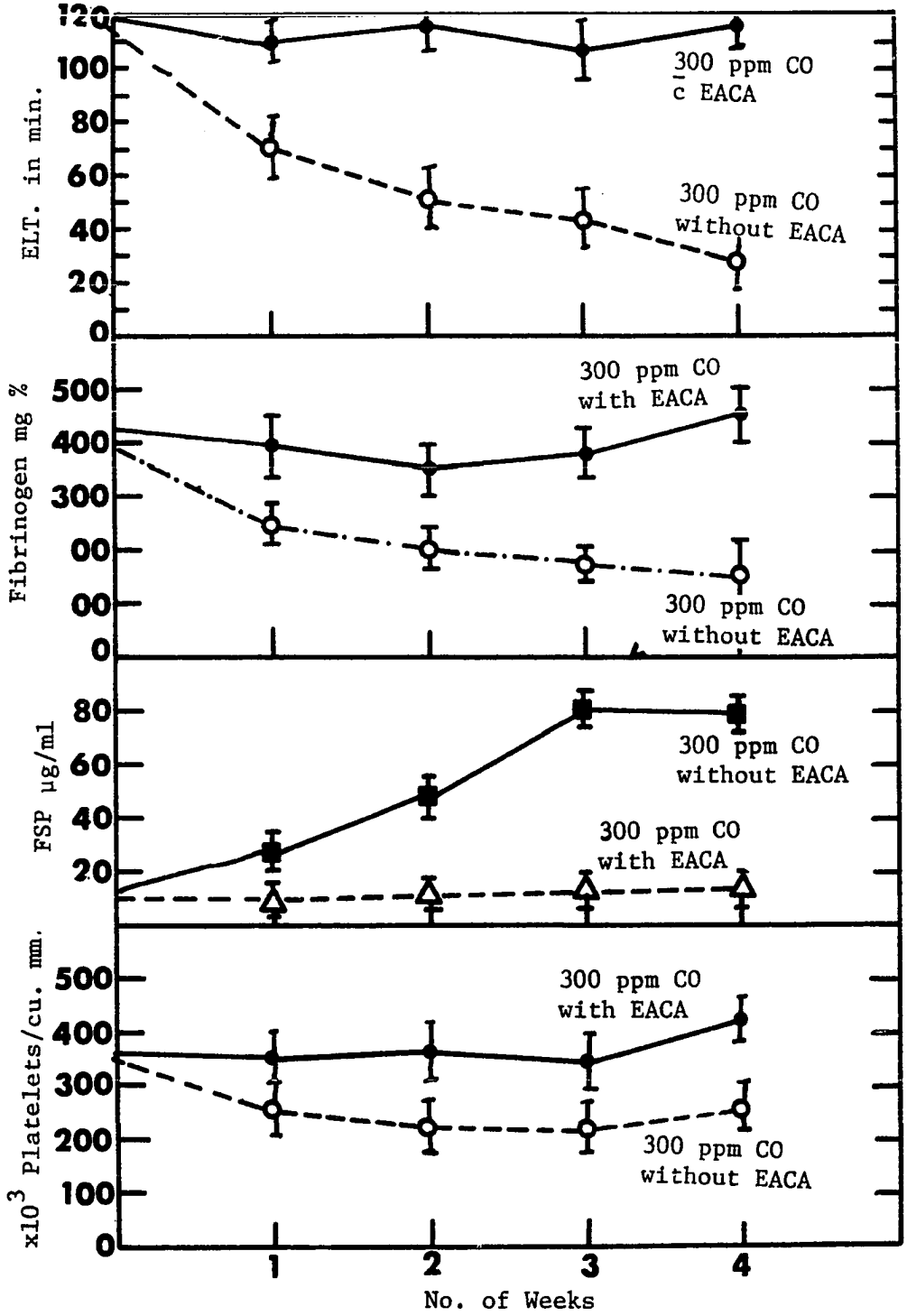
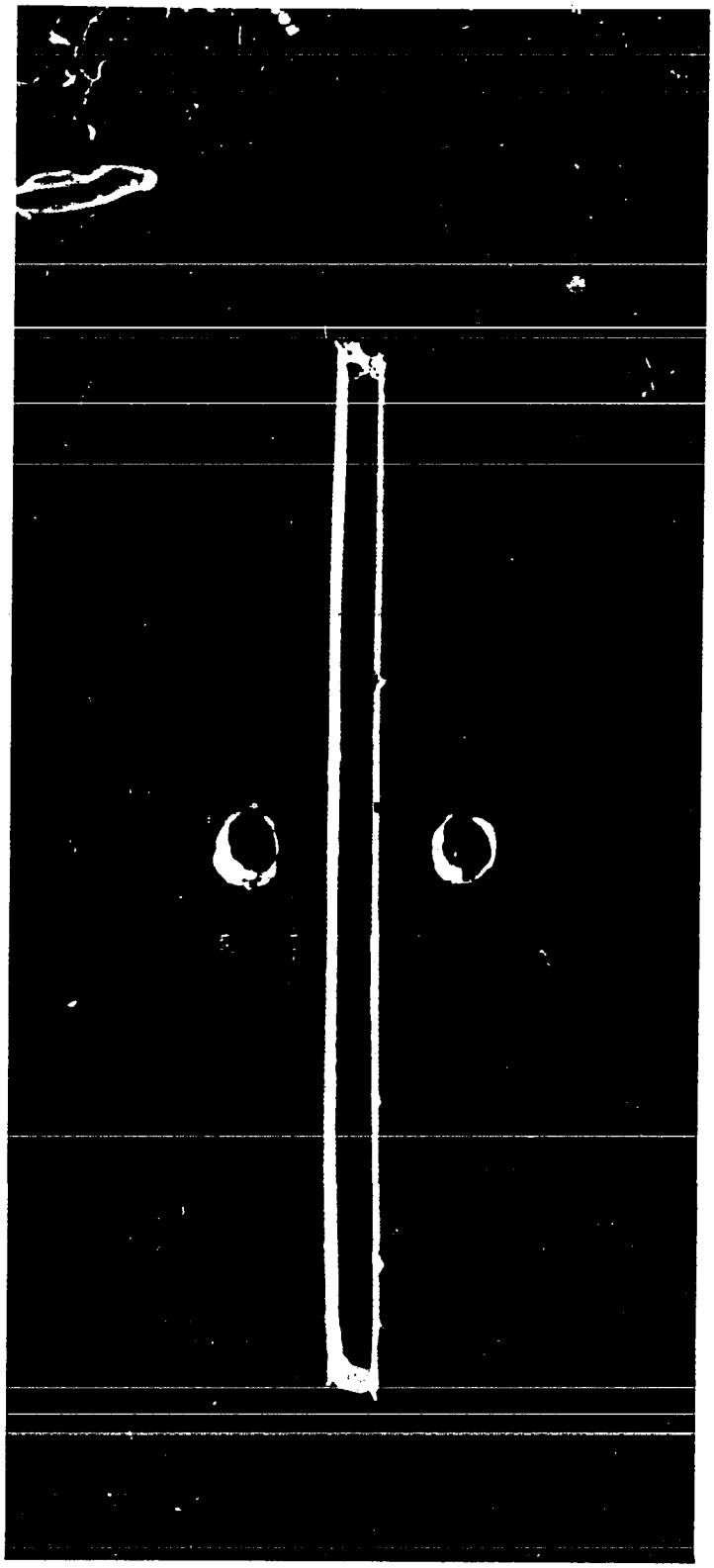

ELT. in min.
120
100
80
60
40
20
0
300 ppm CO
c EACA
300 ppm CO
without EACA
Fibrinogen mg %
500
400
300
00
00
0
300 ppm CO
with EACA
300 ppm CO
without EACA
FSP µg/ml
80
60
40
20
300 ppm CO
without EACA
300 ppm CO
with EACA
x10³ Platelets/cu. mm.
500
400
300
200
100
0
300 ppm CO
with EACA
300 ppm CO
without EACA
1
2
3
4
No. of Weeks

FIGURE 28. Immunoelectrophoresis of urokinases and concentrated urine: Well A is filled with 2 µl of concentrated urine collected from rabbits, well B is filled with 1 µl urokinase (Calbiochem) 100 CTA units/ml, migration proceeded for 45 min. at 250 volts; the troughs were filled with rabbit anti-human urokinase antiserum.



all parameters. These findings were clearly demonstrated that the stimulus of release of fibrinolytic (plasmin) activating substances under the influence of environmental stimulus such as CO is a pathophysiological and physico-chemical reaction which deserves further exploration. The recovery of "tissue" or endothelial activator offers a unique opportunity to study the molecular structure of this enzyme protein and metabolic mechanisms controlling its synthesis, release and degradation.

CHAPTER IV

DISCUSSION AND SUMMARY

Experiments were performed to demonstrate the effect of chronic low level carbon monoxide exposure in rabbits on blood fibrinolytic activities.

Formation of fibrin is an important stage in hemostasis, thrombosis and tissue repair because the fibrin formed solidifies the hemostatic platelet plug and provides a matrix for the formation of reparative connective tissue with fibroblastic proliferation and growth of capillaries. Ultimately, the fibrin has to be removed, mainly by the process fibrinolysis, in order to restore normal conditions. Fibrinolysis is caused by the blood proteinase, plasmin. Plasmin (fibrinolysin) is formed from a precursor, plasminogen (profibrinolysin), present in blood plasma. Plasminogen is transformed to plasmin by a variety of activators (see Figure 1). Inhibitors regulate the activation of plasminogen as well as the effect of plasmin. In the living organism, a dynamic equilibrium, or hemostatic balance, exists between fibrin formation and resolution (see Figure 1). Disturbances in this balance may lead to impaired or to excessive fibrin formation and, consequently, to impaired hemostasis or pathological tissue repair. The involvement of the mechanisms of blood coagulation and fibrinolysis in the regulation

of tissue repair signifies them as fundamental physiological processes, important not only in hemorrhagic disorders and in thromboembolic disease but playing a role in a multitude of pathological conditions.

The fibrinolytic activity of tissue is mainly caused by plasminogen activator. Plasminogen activator is present in normal blood in only small amounts. Most likely it is released from the vascular endothelium. The fibrinolytic system controls and regulates the enzymatic degradation of both circulating fibrinogen and intra- and extravascular deposits of fibrin.

The extent and ultimate fate of a fibrin deposit depends on the degree of injury and on the availability of components of coagulation and fibrinolysis and their inhibitors.

An enhancement of blood fibrinolytic activity in the blood of CO exposed individuals had been previously suggested by Sairo et al., (1968).

In order to understand the effect of CO on fibrinolytic activity in depth using the most advanced techniques and modifying the traditional ones to measure the fibrinolytic activity in highest sensitivity in rabbit blood that was exposed to low-level CO. Traditional studies of CO toxicology have emphasized the dramatic effects of heavy exposure, remarkably little information is available about the consequences of exposure to the low concentrations found in the polluted community air. The concentration of CO chosen for this study was a level to which animals could be exposed

without manifesting an acute CO toxicity and a level below that normally encountered by man in his environment (200-300 ppm of CO inhaled in cigarette smoke) and present for brief periods in confined areas of automobile congestion.

A consistent pattern of acquired change in fibrinolytic activity was observed in the CO exposed animals for the various time intervals, each of which tend to confirm the other. There is little experimental evidence available in the literature to support the claims incriminating CO as a distinct health hazard at the lower levels of exposure. The results of this study suggest that low levels of CO may play an important role in fibrinolytic activity and coronary thrombosis. The effects of CO exposure as reported herein, hopefully, will serve to stimulate further investigation in this area.

The possibility that CO exposure influence the hemorrhagic disorders and the thromboembolic disease in man. This study of the blood fibrinolytic activity under the stimulus of CO exposure will help the future investigators to understand the relation between low level CO exposure and blood fibrinolytic enzyme systems. Under the conditions of these experiments CO did not alter the effect of α_2 macroglobulin. This is very important information for the immunological studies of the blood diseases. In all these studies and statistical analyses, no change was revealed in the quantity of hemoglobin.

The results of this study shows that platelet quantity changes in the low level CO exposure. This is a significant change in the blood of animals. Platelet counts were performed using plasma from animals

that were exposed to CO and received epsilon amino caproic acid (EACA) varied etiology from normal subjects and with those exposed to only CO without EACA. Studies of the effects of CO on blood platelets are contributing to the yet fragmentary understanding of the mechanism of reduction of platelet in the circulation. The number of platelets in blood of CO exposed animal diseases after the depletion of oxygenated hemoglobin begins after about a week of moderate exposure. These results demonstrate that the number of circulating platelets has a regulatory effect on fibrinolytic activity. Moreover, the number of megakaryocytic mitotic figures begins to increase rapidly about 24 hours after platelet depletion, suggesting an influx of DNA-replicating cells into the recognizable population. It is difficult to state with certainty from the present data here that the CO also may cause destruction of some platelets during their maturation process, because data derived from peripher platelet counts may not have precise quantitative value, a marrow study may be needed to understand the cause of platelet destruction.

The low platelet count seen in CO exposed animal blood may be the result of both increased platelet destruction and/or relatively reduced platelet production. Platelet consumption occurs either as part of a process of intravascular coagulation (McKay, 1968) or by the effect of an abnormal vascular surface which is independent of coagulation (Harker, 1970). In the process of fibrin formation, the clotting factors fibrinogen, prothrombin, V, VIII, and XIII are consumed, along with platelets. Widespread vascular injury, i.e., vasculitis, produces thrombocytopenia due to the direct consumption of platelets by the

damaged endothelium, without associated clotting factor depletion. Endothelial damage is considered the etiology of the frequent thrombocytopenia and the less often observed intravascular coagulation.

Platelet consumption is the most likely explanation for the slightly reduced platelet survival observed in atherosclerosis (Murphy and Mustard, 1962) arterial hypertension, hypoxia, thrombo-embolic disease (O'Neill and Fikkin 1964). Reduced life-span of platelets has been observed in gout (Murphy , et al, 1963) acute fibril disorders (Abrahamsen, 1968) and in smokers (Murphy and Mustard, 1963). This brief information about the platelet reduction in blood of CO exposed animals will help the scientists in this expanding field which has developed in the areas of nuclear medicine, cytology, and statistics. Radioisotope techniques were the first to appear, providing in the late fifties platelet labels whose binding characteristics have been thoroughly studied and perfected in the following twelve years. The best of labels enable determination of platelet life-span, pooling and production. The use of radioactive iron to measure megakaryocytes was another recent and fruitful application of radioisotopes to platelet kinetics.

It has been observed that rabbits exposed to low levels of CO were endowed with greater capacity to increase fibrinogen degradation products in the circulating blood.

The t-test was used as a test for significance between the eight-week mean values of both the exposed and control rabbits' serum. The results of these tests were highly significant. Rabbits exposed to low levels of CO have high level of fibrinogen/fibrin degradation products.

The results of the studies associated with decreased fibrinogen concentration indicated that CO, under the experimental conditions employed, served to enhance the release of plasminogen activator from the vessel endothelial into the circulation. The biological activator of plasminogen is a proteolytic enzyme which is capable of hydrolyzing arginine and/or lysine bonds. The type derived from vascular cells is soluble and presumably diffuses into the circulation in response to vasoactive stimuli caused by CO toxication. The release plasminogen activator acts on circulating plasminogen and converts it to plasmin which is an endopeptidase that hydrolyzes susceptible arginine and lysine bonds of fibrinogen or fibrin at a natural pH, producing smaller molecular weight of fibrinogen degradation products. Degradation products of fibrinogen and fibrin appear in the systemic circulation as a result of this enzymatic digestion of circulating fibrinogen or fibrin clot dissolution. These derivatives have striking anticoagulant effects and, when present in sufficient concentrations, contribute to the severe hemorrhagic disturbances seen in the myocardial tissues of exposed rabbits under the microscope.

There is much information available concerning the effect of fibrin split-products upon coagulation systems. The recent report of Barnhart and her associate of the increased platelet aggregation and adhesiveness caused by fibrin split-products also gives an excellent review of some other aspects of split-products on the clotting system.

As previously described, decreased plasminogen activator activity in the blood was demonstrated in rabbits of exposed to 300 ppm CO with EACA. Decrease and total absence of plasminogen activator activity in

these animals clearly explained the possibility of fibrinolytic enzymes being released from the vascular wall. Kwaan and McFadzean showed increased fibrinolytic activity in blood withdrawn from an ischemic arm.

Because of the degradation process in the blood of exposed rabbits, the fibrinogen constant is highly reduced.

Urine samples collected from both groups and checked for fibrinolytic activity on fibrin plate and caseinolytic assay showed no difference in fibrinolytic activity in both groups of exposed and non-exposed (control). Both groups showed fibrinolytic activity on fibrin plate and caseinolytic assay due to the urokinase presence in urine.

The t-tests were used as a test for significance between the mean values of both the test and control groups. This statistical analysis did not reveal any significant difference in fibrinolytic activity between the two groups, the test and the control groups. The fibrinolytic activity is enhanced in the blood of exposed rabbits and the statistical analysis revealed a highly significant difference in fibrinolytic activity between the test and the control groups. These results demonstrate that plasminogen activator is released from the vessel walls of endothelial tissues which is not related to the urokinase activity. Plasminogen activity is enhanced in the blood of chronic low level CO exposed rabbits because of the possible vascular injury, i.e., vasculitis and/or stimulus of the endothelial cells causing the release of plasminogen activator into the circulation.

Several investigators have suggested that the kidney is the source of circulating plasminogen activator (Buluk, et al., 1962a;

Holemans, et al., 1965b; Menon, et al., 1968c) and urokinase (Charlton, 1966a; Nowak, 1966b; Vreeken, et al., 1966c) as well. As for urokinase, the alternative suggestion that this activator is filtrated from circulating blood into the urine has also been reported (Kaulla, et al., 1958a; Smyrniotis, et al., 1959b; Riggenbach, et al., 1961c)

In this present investigation, a significant increase of fibrinolytic activity in blood of CO exposed animals but no change in urokinase excretion was demonstrated. The statistical analysis did not reveal any significant difference in urokinase excretion between the test and control groups. From the results of this investigation it appears to be at variance with the previous assumption that the kidneys are a main source of circulating plasminogen activator. The different interpretations can be reconciled if one assumes that the kidney, instead of being a site of plasminogen activator synthesis as such, is a main organ able to synthesize a "humoral factor" which in turn stimulates the release of plasminogen activator from organ or organs where it is really synthesized.

The question arose whether a high level of non-protein-nitrogen will interfere with the fibrinolytic enzyme system. Norman (1957) had demonstrated that only a urea concentration higher than 1.5 M could inhibit plasmin proteolytic activity on a casein substrate in the test tube. He also demonstrated that normal urine which contains 0.3 M urea induced lysis areas on unheated fibrin plates.

Some investigators suggested that the kidney would excrete activator, partly into the blood stream, and partly into the urine as urokinase. The decrease of both circulating fibrinolytic activity and

urokinase excretion seen in renal disease, would then be easier to understand. However, it has turned out that the relationship of kidney to that fibrinolytic system is not that simple. The observations on fibrinolytic system in exposed and non-exposed rabbits blood as described earlier, were obviously not in accord with the concept of the kidney as the main source for the circulating plasminogen activator.

A basic query permeates all these considerations, namely whether or not urokinase is filtered from circulating blood. Statistical analysis did not reveal any relationship between blood fibrinolytic activity and urokinase excretion in normals as reported in the literature (Charlton, 1966a; Nowak, et al., 1966b) and found in this present investigation as well. This non-existence of this relationship cannot be used as evidence against the assumption that urokinase is filtrate from the circulating blood. First, urokinase might--at least theoretically--be present in the blood as an activator-antiactivator complex, or adsorbed to blood corpuscles such as erythrocytes. Second, the question arises whether a single determination of euglobulin lysis time and the daily urokinase excretion are comparable. Fearnley (1965) had shown that there is a diurnal variation in the fibrinolytic activity of normal persons.

If urokinase is derived from the circulating blood, indeed, by what mechanisms will it be excreted through the kidney? The molecular weight of urokinase is 54,000, smaller than albumin (69,000). It has been shown that the pore radius of the glomerular membrane of the kidney is in range between 18 and 50 A^{o} and serum albumin has a "dimension" of 18 by 75 A^{o} (Norman, 1957). Serum albumin is found in the glomerular

filtrate and is partially reabsorbed by the renal tubule. Consequently, it is possible that urokinase can be filtered through the glomerular basement membrane. Another possibility is that tubular excretion of urokinase from blood into urine. Finally, urokinase might be formed in the kidney. If urokinase is a product of the kidney, one has to explain the demonstrable lag period in those transplanted patients who pre-operatively had no urokinase excretion.

Euglobulin lysis time test, used for the check of fibrinolytic activity of the blood of test and control groups. This test is based on the precipitation of fibrinogen and the fibrinolytically active components of plasma by acidification and lowering ionic concentration. The test has also been modified for use with whole blood instead of plasma. The advantages are that it is relatively simple and can be performed without elaborate equipment. The t-tests were used for the test for significance between the eight week mean values of both the test and control populations. The results of these test presented (Figure 6). By establishing the level of significance to be accepted at 0.05 or 95 per cent, using the eight week mean values of both the test and control population as groups gave a level of significance $P > 0.001$, a highly significant. The third group of rabbits (pilot study) receiving (EACA 200 mg/kg) with 300 ppm CO did not show any significant increase in fibrinolytic activity. The other group of 3 rabbits were exposed to 300 ppm CO without EACA showed enhanced fibrinolytic activity. There is a visible difference in the blood of rabbits exposed 300 ppm CO and the rabbits received EACA with 300 ppm. Whole blood clot of both groups

show a visible difference in their solubility. The clot of whole blood from rabbits that received 300 ppm CO without EACA start lysis after a half-hour incubation at 37°C water bath and in one hour period, 70-80 per cent of the clot lysed. The clot of whole blood from rabbits that received 300 ppm with EACA did not show any sign of lysis (Figure 23).

Determination of alpha-1-antitrypsin concentrations of both the test and the control groups plasma showed no significant differences between the exposed rabbits' plasma and the controls'. The t-tests were used as a test for significance between the eight-week mean values of both the test and control populations at the level of significance to be accepted at 0.05 or 95 per cent, analysis did not reveal any significant difference between the test and control groups.

The α_2-macroglobulin test is used to determine the quantity of α_2-macroglobulin in the blood of test and control rabbits. Statistical analysis did not reveal any significant difference between the test and the control population. From these last two observations (alpha-1-antitrypsin and α_2-macroglobulin tests) it is clear that increasing plasma plasminogen activator in the blood of CO exposed rabbits does not appear to be affected by these inhibitor proteins. Relationship between plasminogen activator and these inhibitor proteins are known to a certain extent, but it is not clear that the increased plasminogen activator induces the release or synthesis of these proteins. There were no experiments carried out during this present investigation to probe into the possible mechanism of removal of the plasminogen activator from the circulation. Several investigators have suggested that the liver is clearing the plasminogen

activator from the circulation. There is no doubt that the liver clears plasminogen activator, but, the exact mechanism by which this is achieved needs much further investigation. It would not be surprising if future studies should show that the liver is capable of the triple functions of excretion plasminogen activator with the bile, abolishing plasminogen activator of hepatic blood flow and being able to control the release of plasminogen activator from organs or tissues as well.

The fibrin plate method assay used for the measurement of plasma fibrinolytic activity of the exposed and control groups. A reference standard curve was constructed and using the reference curve the activity was converted to CTA unit of urokinase per ml.

The fibrin plate (Hyland) showed a significant difference between the test and the control groups. There were visible (Figure 23) and obvious differences between exposed rabbit plasma lysis zone and control rabbit plasma lysis zone. Essentially, control rabbits' (non-exposed) plasma did not show any lysis on the fibrin plate after 16 hours of incubation at $37^{o}C$.

Modified Micro-Kjeldahl Method used for fibrinogen determination in the plasma of the rabbits exposed to CO and non-exposed (control). The t-tests were used as a test for significance between the eight-week mean values of both the exposed and control rabbits' plasma. The result of these tests were highly significant. Rabbits exposed to CO have lower concentration of fibrinogen. The clottability test for fibrinogen gave the similar results as Kjeldahl.

Caseinolytic assay on exposed and nonexposed rabbit plasma: A

typical result from caseinolytic assay shown in (Figure 23). Increased caseinolytic activity is observed from the plasma samples of exposed rabbits. The activation reaction followed zero order kinetics for the first 20 minutes with the rate of plasmin generated being roughly proportional to the amount of activator present. EACA (20mM) in the activation mixture completely suppressed the plasminogen activation by the exposed rabbit plasma. At the zero time, small amounts of activations were found and were probably due to the plasmin contamination of the plasminogen preparation. This blank value was subtracted. The evolving plasmin activity, therefore, is solely the result of plasmin generated during the incubation period. Here again, the reaction followed zero order kinetics for the initial period and the initial rate of plasmin generation was proportional to the activator concentration.

Microscopic findings in myocardial tissues were as follows: the capillaries are extended filled with red blood cells which reveal clumping. In some areas, there are slight extra-capillary accumulation of the red blood cells. In other areas, the arterial seems to have a thickened wall, and initiation of infraction is seen, in the myocardial tissues of exposed rabbits. Hardening of arteries and deposits of fatty material, chiefly cholesterol within the inner walls of arteries were seen (Figure 23 a,b,c.)

Summary and Conclusions

Traditional studies of CO poisoning have emphasized the dramatic effect of heavy exposure, but little information is available about the low level chronic exposure of CO found in community air. The small amounts

of carboxyhemoglobin in the blood has so far not been regarded as having a significantly harmful effect. It is hoped that the results of this study will convince that the previous assumption is wrong.

In man, intermittent exposure to carbon monoxide, rather than to nicotine, due to tobacco smoking may be regarded as the real cause of much higher risk for smokers to develop arterial diseases compared with non-smokers.

The chronic low level CO exposure causes deposition of fatty material on the endothelial of the vessel walls that causes tissue damage because of lack of oxygen. This tissue damage results, often producing death by heart attack, strokes, etcetera. The medium-sized arteries supplying the heart, brain, extremities, and other organis with blood becomes increasingly less able to furnish enough blood to these organs because of decreased size of the lumen, or inner diameter of the blood vessel.

Increase in permeability of the endothelium tissues induced by CO or hypoxia leads to the formation of subendothelial edema, lipid accumulation, and other arterial injuries seen in experimental animals. This agrees well with the filtration theory for the pathogenesis of atherosclerosis and emphasizes the importance of this theory. The molecular process involved in the permeability change should be identified and a possible relation to a hypothetical oxygen-dependent control system for endothelial permeability should be evaluated.

Change in endothelial permeability is not only causing increased fibrinolytic activity but possibly increased permeability of other signi-

ficant enzymes in excess into the circulation.

Increasing fibrinolytic activity may cause a great change in viscosity of the blood flow which is very important factor in coronary heart diseases.

Increase in carboxyhemoglobin may change the synthesis of several enzymes and clotting factors. A drop in the level of the clotting factors II, V, VII, and IX (except fibrinogen and X) in the liver certainly can contribute to the bleeding diathesis in the chronic CO exposed animals.

COHb levels measured after smoking may indicate the risk for the smoker in the work place or in the community might help to discourage smoking.

The result of this investigation suggests that the CO concentrations in occupational exposure to CO concentration not greater than 20 ppm was determined as a time-weighted average (TWA) exposure for an 8-hr per day.

In evaluating the exposure of employees who have coronary heart diseases with angina pectoris to CO sufficient to produce a COHb level of 5 per cent, while a small additional decrease in O_2 saturation of the blood brought about by mild exercise might be feasible, "the degree of blood oxygen desaturation demanded with 10 percent COHb loading is rather 'severe'." The recommended TWA standard of 35 ppm CO is based on a COHb level of 5 percent, which is the amount COHb that an employee engaged in sedentary activity would be expected to approach in eight hours during continuous exposure. The recommended standard does not take into consideration the smoking habits of the worker since the level of COHb is

chronic cigarette smokers has generally been found to be in 4 to 7 percent range prior to CO exposure. The recommended standard is based on the utilization of the Coburn equation to predict the mean COHb level of non-smoking employees exposed to a known TWA concentration of CO for an eight-hour workday. The applicability of the equation for this purpose has been validated by a study of Peterson and Stewart in which the COHb levels of sedentary young males exposed to known TWA concentrations of CO for known periods of time were predicted by the equation.

The investigations leading to the results presented here have proved fascinating and challenging to us, not only because of their important practical aspects concerning a disease which today dominates the mortality statistics in this part of the world.

Research in the coming years will show if new developments and new ideas of importance for the theory and practice of environmental control.

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