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INVESTIGATION OF MANGANESE BINDING POTENTIAL
IN NEOPLASTIC AND NON-NEOPLASTIC TISSUE

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Preface

Man has always been plagued by disease. In the opinion of this author, it is foolish of man to detest disease as the causative agent (be it a cancer cell, parasite, etc.); it is simply an entity fighting for survival, as is man. In the fight for survival, the individual man often forgets his fellow man and is in many instances responsible for his demise. Such being the case, has man any right to be critical of a disease-producing organism?

However, one lifetime goal of this author has been to aid in alleviating mankind's suffering from disease. It is hoped that this dissertation makes some contribution in the achievement of this end. The credit for this work should not go primarily to the author, his mentor, Rena (who tirelessly slaved so many nights in the laboratory), Dr. Donald Clarke (for his advice on carcinogenesis), Mrs. Ellen Wildman (who taught this author far too many things to be mentioned here) or to Dr. Ruth Kleinfeld (who so kindly performed liver resection for us). Although the help of the above was invaluable, we wish to acknowledge Samuel and Leah Zimmerman who died on June 27, 1966 and June 8, 1965, respectively. It was their unceasing love for their fellowman that was tantamount in making this work possible.

Abstract

In an attempt to discover methods of controlling cancer, two basic philosophies of research prevail. The first involves testing vast multitudes of drugs in the hope that one may be found which destroys the malignancy without killing the patient. The second espouses the finding of differences between neoplastic and non-neoplastic tissue in the hope that these differences may elucidate the nature of the carcinogenic process. Once this is known, one would hope to be able, teleologically, to select possible methods of cure. Clearly, a great number of differences between neoplastic tissue and the tissue of origin of the neoplasm simply reflect cellular differences and in no way relate to the carcinogenic process.

The dissertation will be concerned with the differences in manganese binding potential in neoplastic and non-neoplastic tissue that has been observed by the use of Electron Paramagnetic Resonance (EPR). A general discussion of EPR and the role of manganese in normal tissue will be followed by a proposed theory of carcinogenesis. Evidence will then be presented for differences in manganese binding potential between neoplastic tissue and the tissue of origin for six different systems. Competition studies will be utilized

to show that the effect observed with manganese cannot be seen for a variety of other ions (i.e., it is not a common ion effect). A brief discussion on endogenous manganese levels will be included. A discussion of purification of the binding system will follow.

In conclusion, data will be presented regarding manganese binding potential with respect to the carcinogenic process.

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INTRODUCTION

Cancer--Its nature, history, and relationship to life

There have been many attempts to define the disease cancer. A good many of these definitions reflect the particular dogma of the author with respect to causation of the malady. As an example, Berglas (9) defines cancer as "a runaway healing attempt". Warburg considered cancer to be composed of masses of cells in which the tricarboxylic acid cycle was somehow damaged. Clearly, until cause has been definitely established, we can at best suggest a very loose definition based on morphological and visual observations. One might suggest cancer be defined as a mass of partially or wholly undifferentiated cells in a state of uncontrolled proliferation probably originating from some tissue in the host and resembling the cells of that tissue to a greater or lesser extent. Certainly, a definition as loosely structured as this leaves much to be desired. Yet, to attempt to formulate a more structured definition means making certain assumptions which may or may not be true. For example, if one implants a crystal of a carcinogen such as 20-methylcholanthrene in the liver and develop a tumor of that organ some time later, one can be fairly certain that the carcinogen in some way caused liver cells to become malignant. Yet if one observes a spontaneous hepatoma,

unless it is noted that the neoplastic cells have morphological characteristics of the liver cells, one has little more than intuition to assume that these cells are derived from liver, rather than any other tissue in the body. Hence, one cannot say as a fact that a given cancer cell, although found in a certain tissue, actually originated there. In like manner, the other rather nebulous points in the definition could be so analyzed.

The point of the above semantic discussion is to emphasize that in analyzing any research on cancer, it must be remembered that the author (no matter how objective he may be) has certain preconceived notions on cancer causation. If the data he obtains fits his theory, he explains it in that manner. Yet, due to how very little we know about cancer, that data may later prove to mean something entirely different. With respect to this dissertation, certain ideas are suggested as to how manganese binding potential may relate to cancer and cancer causation. It is hoped that too broad generalizations for the facts presented have been avoided.

As Medes and Reimann (43) point out, cancer has most probably been in existence for a much longer time than man. They cite evidence of tumors existing as much as eighteen million years ago (from fossils). The first record of cancer in man was seen in the Ebers Papyrus

estimated to have originated at approximately 1550 B.C. As time has progressed from that date to the present, the number of reports of occurrence, cause, cure and the widely varied mele of subjects pertaining to the disease have increased geometrically. There are many excellent reviews (e.g., 44, 54, 60) on this subject.

Cancer has been called by many "a disease of civilization". Whereas over the period of time during which man's knowledge increased, many of the maladies that plagued him such as typhoid fever, smallpox, and tuberculosis decreased both in occurrence and human fatalities recorded; cancer (as well as heart disease) have shown opposite trends. There is indeed a great deal of evidence that cancer is "a disease of civilization", if one considers some of the problems we have encountered in our "becoming civilized". (One must point out, however, that the last word in the preceding sentence to many refers simply to more advanced technology and has no relationship whatsoever to increased morality.)

One may cite several concrete examples that suggest why cancer has been increasing over the years.

The first example of carcinogenesis (cancer causation) by chemical substances was observed in the eighteenth century by Sir Percival Pott (52). He recorded the fact that chimney sweeps had a high incidence of cancer of the

scrotum and suggested that this might be due to the soot which accumulated on their clothes during work. The hypothesis remained untested until 1918 when Yamigawa and Ichikawa (68) succeeded in producing cancer on the ears of rabbits by the painting of coal tars. Following publication of this work, there were many other studies which showed that coal tar could induce cancer. Even so, it wasn't until 1933 when Cook et al (14) isolated the first pure carcinogenic hydrocarbon from coal tar. This compound was 3,4 benzpyrene. Since that time, a wide variety of cancer causing compounds have been discovered. By 1941, Hartwell (28) reviewed over 170 compounds that were found to be carcinogenic. The texts of Greenstein (25), the Daudels (15), and Clayson (12) offer detailed discussions regarding currently known carcinogens.

Since most of the work on carcinogenesis has been performed on animals, there are some (notably members of the tobacco industry, etc.) who question whether these compounds can cause malignancy in man. Even though experiments involving carcinogenesis on humans are rather unacceptable, there are distinct indications that man is also susceptible. For example, Kennaway et al (33) noted a distinct correlation between occupation and various forms of cancer. In each instance cited, the workers were exposed to substances containing carcinogenic compounds.

As another case in point, it was found that workers in Basel, Switzerland using coal tar dyes developed bladder cancer in a comparative ratio to the rest of the population of 33:1 (31, 46). It was also found that beta-naphthylamine found in these dyes was quite active in inducing bladder cancer. Studies citing many other such correlations are available.

The primary question that now must be raised is whether, when a large number of men are exposed to carcinogenic compounds, any correlation with number of cancer cases is observed. Two primary examples of this are the correlation in cases between city and country dwellers and between smokers and non-smokers. In the first case, it is apparent that city dwellers (in most instances) are exposed over long periods of time to industrial wastes (i.e., air pollution) whereas the country dwellers (with the exception of heavily industrialized towns) are not. Naturally, industrial interests seek to prove that no such relationship exists. However, many studies indicate the strong possibility of such correlations. For example, as early as 1942, Leiter and coworkers (39) showed that extracts of city dust gave sarcomas when injected into mice. It was found by analysis of London fog (9), that carcinogenic hydrocarbons were present. Concentrates of substances found in air in

Los Angeles when painted on mice gave malignancies in 30% of the cases (37). More detailed discussions are presented in the texts of Greenstein (25), Berglas (9), and Ambrose and Row (5).

In the example of tobacco usage, the correlation between exposure of man to compounds known to be carcinogens in animals and incidence of cancer is even more striking. The fact that tobacco smoke contains carcinogenic compounds is well documented (e.g., 3, 4, 12, 67). Some (mostly propagandists from the tobacco industry) claim that many compounds normally in use could be considered carcinogenic if given in large enough doses; hence cigarettes are no more dangerous than most products available today. The fact that cigarette smoke has been shown to contain 3,4 benzpyrene (12), one of the most potent carcinogens known, invalidates this argument. Hence, since one knows that cigarette tars contain small amounts of potent animal carcinogens, if these compounds are active in man one could expect a greater incidence of lung cancer in smokers than non-smokers. This has been shown to be the case (9, 12, 61). Many fine pamphlets available from the American Cancer Society further support this contention.

It is felt from the above that it is clearly indicated that cancer is "a disease of civilization". Also, the

potent animal carcinogens probably do affect man (although exposure time and dosage are assuredly greater).

As an added point of interest, one might suggest an answer to the question of why if cigarettes contain potent carcinogens are they not banned. It appears that in instances where a large industry will suffer financially from a product being banned from sale, those responsible for the decision require absolute proof. Cigarettes are not the only product in which the public has been exposed to potent carcinogens. For example, it is well known that Butter Yellow (4-Dimethylaminoazobenzene) is a potent liver carcinogen (12, 15, 25). Yet for many years the margarine industry was permitted to enclose small packets of butter yellow with white margarine so as to improve the appearance. As another example, the fact is cited that no manufacturer of hair dyeing products is required to submit to anyone (including the government) a list of the compounds contained in their preparations. Many of the aniline dyes are known to be carcinogenic and no one has any way of knowing which, if any, of the preparations contain these. There are many other examples that could be cited (e.g., 9). As a parting word on this subject, a special report of the National Research Council of the National Academy of Science (47) is quoted. "It may take the Food and Drug Administration another 25 years to

determine whether 116 dyes now certified as safe under the law really are harmless."

The purpose of the rather general discussion in the past few pages of this dissertation has been two-fold. First, an attempt has been made to point out a few aspects of the scope and problems involved in cancer research. And second, it was hoped to suggest reasons why cancer is so different from other maladies and why as man progresses it becomes a bigger problem. To cure typhoid is simply a matter of destroying the bacillus Salmonella Typhosa, a creature alien to man. Yet to cure cancer, a disease springing from man's own cells, one is greatly plagued by problems brought on by the host himself.

A Proposed Theory of Carcinogenesis

A great many theories of carcinogenesis have been proposed. Any attempt to discuss these would certainly contain many omissions if for no other reason than for lack of space. However it can be seen that carcinogenic theories can be divided into two large classes. The first involves speculation as to the nature of the agent or agents responsible for the neoplastic transformation. The second class attempts to define the cellular process or processes that are affected by the primary agent(s).

On the basis of the results that will be discussed in the remainder of this dissertation, the following theory

of carcinogenesis (i.e., of the second class above) is presented.

1. There exists, as a normal cellular component, a specific system for binding the ion manganese.
2. The system consists of two sub-systems: a) binding (proven) and b) inhibitory to binding (indicated but not proven).
3. The binding sub-system consists of two components: a) a non-protein cofactor and b) a low molecular weight polypeptide (possibly conjugated).
4. The purpose of the system is to regulate the amount of manganese available to manganese-requiring enzymes in the cell and hence act as a control system. (Mn bound to the binding sub-system cannot be utilized by the cation-requiring enzymes.)
5. Neoplastic tissue has undergone some type of alteration whereby the binding system cannot function as efficiently as in non-neoplastic tissue.

Points 1-3 are clearly indicated by the results presented in this dissertation. Point 5 suggests a change during the carcinogenic process. The experiments presented with regard to manganese binding potential during the carcinogenic process suggest the validity of this point. However, it should be pointed out that a completely

controlled carcinogen experiment would involve, aside from the controls that have been performed, measurement of carcinogen in the tissue taken at each time of testing as well as removal of only part of the organ from each individual animal with the animal not being sacrificed so as to determine the length of time for neoplastic development in each case. The physical limitations of such an experiment would of necessity make it exceedingly long term. Such an experiment is planned for the near future. It is felt that the three carcinogen experiments reported herein do yield preliminary evidence with respect to point 5. This is primarily because of the very similar pattern of manganese binding potential versus time that emerges in all three cases.

Point 4 is simply a plausible explanation of the results. Although in the near future several experiments with specific enzymes of carbohydrate metabolism will be performed in order to establish whether this point is valid, the experimental data contained in this dissertation gives no evidence to support it.

It should be stressed that the above represents a theory in its most preliminary stages. It has been proposed as such, rather than simply reporting the results with a less provocative interpretation, in the hopes that it will stimulate others to work on what it is felt is a very exciting, but relatively unexplored field. Whether this

theory has validity will be shown, not by the research presented herein, but by future experimentation.

Manganese--Role in Cellular Metabolic Pathways, Growth and Cancer

In this section an attempt will be made to justify point 4 in the theory of carcinogenesis that was proposed. It is rather unfortunate that there have been relatively few investigations on the involvement of manganese with normal metabolism; and even fewer on its relationship to cancer. As to the latter, only four pertinent works on the subject (18, 58, 69, 70) have been detected. These give little indication of what, if any, relationship exists between the ion and the malady. As an example, Zapadnyuk (69) found that injections of $MnCl_2$ or $CuSO_4$ caused a decrease in spontaneous breast cancer (a most variable quantity to measure) in rats.

In this dissertation, the primary concern is manganese binding potential. This can be defined as the ability of a given tissue to bind a known quantity of exogenous manganese per unit of protein in the tissue. (A more complete discussion of this definition will be given later.) A thorough literature survey did not reveal any works concerning the relationship between manganese binding potential in neoplastic and non-neoplastic tissue.

With respect to the relationship of manganese to growth, the literature, although sparse, is more complete than the relationship to cancer. Many studies (e.g., 13, 17, 34, 35, 45, 63) indicate that the ion is important in the growth process. In various plants and animals, several authors (13, 34, 63) found that manganese deficiency inhibited growth, while others found that excesses of the ion caused increased growth (17, 35). It is also interesting to note that Michel (45) found that Mn^{54} administered intravenously was retained longer in the pituitary and thyroid (both important in growth) than in any other organ tested. Polyakova (50) found that manganese deficient diets caused impaired function of the thyroid. In another interesting study, Webb (64) found that certain strains of bacteria needed less magnesium for optimum growth in the presence of excess manganese than they would if the latter were not added. Other bacteria did not show this effect.

The significance of these studies on growth has yet to be determined. However, it appears that manganese does play some role in the growth process.

Perhaps the greatest number of studies conducted with respect to manganese and the life sciences are those on the enzymes that require the ion. Yet even here to get any rational idea on the relationship of Mn to the cellular

metabolic pathways will require a great deal more work. As an example the case is cited of a colleague who found manganese contaminants in a sample of commercially purchased poly guanylic acid. Upon writing to the firm and at our request seeking information on method of synthesis, he was informed that polynucleotide phosphorylase with manganese as the cofactor was the preferred mode. Yet in all of the references located on this enzyme, magnesium is listed as the required cofactor. It would appear strange that a commercial firm seeking optimum yield of product would utilize any but the cofactor that would best attain this end. If manganese is this ion, then it appears somewhat premature to claim Mg to be the cofactor required by polynucleotide phosphorylase, since both ions are normally present in the cell. This would indicate that more study is required on the role of the ion in nucleic acid metabolism.

There have, however, been several interesting studies on this topic. The following are a few examples. It has been found (42) that DNA polymerase in the presence of manganese (rather than magnesium) will catalyze formation of polyribonucleotides as well as polydeoxyribonucleotides. Liao (41) suggests that liver has both manganese and magnesium requiring RNA polymerases. Hsieh (30) claims that E. Coli Q₁₄ mutants contain a manganese dependent

polynucleotide phosphorylase that cannot utilize magnesium as a cofactor. As was mentioned above, it is assumed that normal E. Coli polynucleotide phosphorylase requires magnesium. It has also been found (65, 69) that manganese binds to DNA and increases its stability to heat (34). To attempt any interpretation of these results would be premature. However, the studies do suggest that manganese plays some role in nucleic acid metabolism and ultimately protein synthesis. Studies by Weser (65) and Silva (59) indicate that excess manganese increases protein synthesis in rat livers and tomatoes, respectively.

Other effects of the ion on various metabolic pathways have been studied (e.g., 27, 55, 56), but the only system where one is able to suggest a teleological relationship between manganese and metabolic control is in carbohydrate metabolism.

In figure 1 a rather general outline of carbohydrate metabolism is shown. Those enzymes which either require or are activated by manganese are indicated. The fact that one of the major sites of glycolysis is inhibition of phosphofructokinase by citric acid is well documented (42, 66). Since the enzyme aconitase has its equilibrium in the direction of citric acid, the amount of citric acid buildup is dependent on isocitric dehydrogenase. Although the question of the two isocitric dehydrogenases (i.e.,

TPN dependent and Mn requiring, and DPN dependent and Mg or Mn requiring) is still unsettled, the versatility of manganese in this system would suggest its importance. Another important site of control of carbohydrate metabolism occurs at the junction between glycolysis and the citric acid cycle. It can be seen from figure 1 that the majority of enzymes occurring at this junction are manganese requiring, or are activated by the cation. The fact that the relationship of manganese to many control enzymes has yet to be elucidated can be shown in the case of pyruvic kinase, a magnesium requiring enzyme which has recently been shown by Passeron (49) to be allosterically activated by manganese. It should be pointed out however that the enzyme source was mucor and it is presently unknown whether that is the case in mammalian systems. However, it is interesting to note that Potremoli (51) claims that manganese as well as magnesium can be bound to fructose 1.6 diphosphatase (i.e., the enzyme that reverses the step catalyzed by phosphofructokinase). Although effects of manganese on other enzymes have been studied (e.g., 7, 11, 16, 20, 21, 26, 27, 40, 48, 56), the small number available and the fact that many were conducted in different systems suggests that the only conclusion that can be drawn is that manganese plays an important role at two major control sites of carbohydrate metabolism. With

respect to manganese and the pentose shunt, it is known that the ion is the cofactor for 6-phosphogluconic acid dehydrogenase. A recent study (32) claimed that manganese could fulfill the cofactor requirement of aldolase. Clearly, to elucidate the precise role of manganese in carbohydrate metabolism, more detailed study is needed. But the importance of the ion at two known major regulatory sites of carbohydrate metabolism suggests speculation as to its role in control of high energy compounds. As an example, one might postulate some agent or agents the controlled to amount of manganese available to the critical Mn requiring enzymes of carbohydrate metabolism. Breakdown of this system of agents would cause uncontrolled carbohydrate metabolism which would result in uncontrolled production of high energy compounds (e.g., ATP, DPNH). One might also suggest that a cellular impairment might not result in complete loss of control, but rather loss of a partial nature. Such a cell, although technically damaged, would have a more plentiful supply of high energy compounds available at a given time. One might logically expect such a cell to proliferate more rapidly than a non-damaged cell. Examples of such cells could be neoplastic tissue. Thus, although not enough is known about the role of manganese in the majority of metabolic pathways, the relationship between the ion and carbohydrate metabolism is in line with the theory of carcinogenesis

formed as a result of our research and stated on the previous few pages.

It is stressed, however, that this simply represents a plausible explanation. For the simple idea of manganese control of carbohydrate metabolism to be affirmed or negated, a comprehensive study of the kinetics, thermodynamics and other essential properties of the critical enzyme reactions with manganese as the cofactor must be performed. It is hoped that more interest in such studies (in lieu of the vast multitude involving magnesium) will be generated in the future.

Electron Paramagnetic Resonance--Concepts

The use of electron paramagnetic resonance (EPR) by the biochemist began in comparatively recent times. However, in the past few years biochemical and biophysical experimentation involving EPR has risen at a geometric rate. In this section, the basic principles behind this important technique will be briefly described. For further information, the texts of Ingram (32) and Ayscough (8) are suggested.

Basically ESR can theoretically be used to study any moiety containing an unpaired electron. Thus it becomes a useful tool for the study of paramagnetic ions and free radicals. It must be remembered however that many times it is very difficult in a practical sense to do what is

theoretically possible. The physical state of the molecule, the solvent (if working with solutions), the nuclei surrounding the free electron(s) and many other considerations play a role in determining whether or not an experiment is feasible. It is also true that in many instances, due to the fact that quantum mechanical studies are in such an early stage of development, we really don't know why a given application of EPR should work, but doesn't! However even with these considerations in mind, EPR still offers methods of analysis hitherto unavailable to the biochemist and biophysicist.

Electron paramagnetic resonance techniques are based on the fact that a given electron can have two spins, referred to as plus or minus. The text of Gould (23) presents a simple and concise discussion of the general quantum mechanical picture of the electron. When a compound with an unpaired electron is placed in a magnetic field, some of the electrons will line up with their spins parallel to the field while others are anti-parallel. For an electron to "flip" from one state to the other, the following equation must be fulfilled.

$$h\nu = g\beta H$$

In this equation, h refers to Planck's constant, ν refers to the frequency of radiation necessary to cause the

"flip", g or spectroscopic splitting factor is a characteristic of the given electron under consideration (for free radicals this is very close to the value of 2), β refers to the value of the Bohr magneton and is also a constant, and finally, H refers to the applied magnetic field. It can be seen that if one is studying a given compound (and hence g can be considered constant for the experiment), one can vary either γ or H in order to fulfill the relationship. For engineering reasons, γ (or the frequency of microwave power emitted by the klystron) is held constant while the magnetic field is varied.

Thus when the above condition is fulfilled, electrons in the parallel (low energy) state will absorb energy and attain the anti-parallel (high energy) state, while electrons in the anti-parallel state can lose energy and return to the parallel state. Clearly there must be more electrons in the parallel or low energy state for there to be any net absorbance of energy. Although in general this is the case, in some instances it is not. When that occurs, one may attain the desired distribution by lowering the temperature (for theoretical reasons, see the Boltzmann distribution). Hence, if there is an excess of electrons in the low energy state, upon fulfilling the resonance equation, there is net absorbance of energy which can be detected by an EPR spectrometer. Ingram (32) presents an

excellent discussion on the methods of detection employed in commonly used spectrometers. It should be remembered that since most spectrometers modulate the signal, rather than observing a simple peak, the first derivative trace will be seen.

Anyone familiar with EPR will know that many compounds possessing only one free electron will give more than one peak. This is primarily due to two concepts known as hyperfine and fine interaction.

Hyperfine interaction refers to that between an unpaired electron and a nucleus possessing spin. The nuclear spin, as with the electron spin, will be parallel or anti-parallel to the magnetic field. Now if the nucleus is close enough to the free electron, its effect must be considered. Its spin will create a small magnetic field either parallel (and hence adding to) or anti-parallel (and hence subtracting from) the applied magnetic field. The number of different orientations is based on the spin value of the nucleus and can be calculated from the formula $(2I + 1)$ where I is the value of the spin.

As an illustration, consider a nucleus with a spin of one-half. (If the following discussion is unclear, refer to page 22 of Ingram's text (32).) There are thus two orientations; one parallel and one anti-parallel to the externally applied magnetic field. Now assume one is

dealing with a hypothetical compound with an electron that fulfilled the resonance condition at 4200 gauss. Further assume that the nucleus (with a spin of one-half) closest to the electron produced a magnetic field of 10 gauss. Were this nucleus not present, one would observe one peak arising from the electron "flip" when the external magnetic field was at 4200 gauss. However, if one considers the effect of the nucleus, when the external magnetic field is at 4200 gauss, the effective field acting on a given electron will be 4210 gauss (i.e., its nucleus in parallel to the field) or 4190 gauss (i.e., its nucleus is anti-parallel to the field). Since neither 4210 or 4190 fulfill the resonance condition, an externally applied field of 4200 gauss will no longer yield a peak. However at an externally applied field of 4210 and 4190 gauss, electrons will see a total field of 4200 gauss (if the nuclei are anti-parallel or parallel respectively) and hence a peak will be observed at each of these two points. Thus one can see how hyperfine interaction caused the splitting of one line into two. The number of lines produced by a given hyperfine interaction can easily be seen to be $(2I + 1)$ where I is the spin of the nucleus. The hyperfine splitting constant refers to the distance between the peaks (or twice the value of the magnetic field induced by the nucleus).

Fine structure refers to interactions between unpaired free electrons in a given moiety (most commonly paramagnetic ions). The concepts that were just given for hyperfine interactions also apply here with the one major difference being that the number of lines is determined by the total spin of the electrons and is determined by the formula $(2S + 1)$ where S is that spin.

A final point that must be covered is that of mechanisms by which electrons in the anti-parallel high energy state return to the parallel state. Clearly, there must be methods other than the energy emission previously discussed. Were this not the case, there would soon be equal numbers of electrons in both states and it would be impossible to have a net absorption. Fortunately, there are other methods available and these are termed relaxation processes. There are two of these processes known as a) spin-lattice interaction and b) spin-spin interaction. Spin-lattice interaction refers to the condition where the electron loses energy to a molecule taken as a whole. From this we define spin-lattice relaxation time as "the time in which an initial excess of energy given to the spins will fall to $1/e$ of its value" (32). Hence stronger interaction will produce a shorter spin-lattice relaxation time (known as T_1) and vice versa. The spin-spin relaxation time (referred to as T_2) has the same

definition; but the interaction referred to is between spins of various electrons rather than between the spins and the molecules as wholes. T_1 and T_2 are also referred to as longitudinal and transverse relaxation times, respectively. The width of the signal is inversely proportional to both T_1 and T_2 . Since T_1 is normally greater than or equal to T_2 , it is the latter which is normally primarily responsible in determining line width. For a comprehensive treatise on this subject the text by Bloembergen (10) is recommended.

An important point to remember with regard to the above is that an apparent decrease in amplitude of a given signal may merely be due to an increase in width caused by a decrease in the relaxation time. This point is essential to keep in mind with regard to any quantitative studies on a given species. Rather than observing a change in concentration of the free species after performing a given experiment, one might simply be seeing an increase in line width. One way of checking for this is to measure the area under the peaks obtained from the experiment. If one is observing a decrease in concentration rather than an increase in line width, the decrease in area after the experiment should be proportional to the decrease in concentration. In general, one rarely sees a decrease in amplitude being proportional to decrease in concentration.

However, Leigh (38) proposes a physical model to show how this is possible for the ion manganese.

One simple method of showing that line width changes are not causing a decrease in amplitude in one's experimental system is as follows. This is easily seen by an example. Assume that a given standard subjected to certain experimental conditions has an amplitude of peaks equal to twenty-five percent of that before treatment. If there are no line broadening effects involved in the observed spectrum, one would assume that the final concentration of free species was 25% of the original. Hence, one should be able to dilute the original standard to 25% of its original value and superimpose it upon the experimentally observed spectrum. If one cannot, then line width broadening is involved. It is this method that has been used in this dissertation to show that decrease in amplitude is directly proportional to concentration decrease for the manganese ion in the experiments.

Use of EPR as a Tool to Study Manganese Binding

Electron Paramagnetic Resonance has in recent years been widely used to study structure by binding of free radicals. By synthesizing a given free radical (e.g., nitroxide) with various molecular structures and attempting to react it with biological molecules, one can determine much information about the nature of the structure of the

natural products. As examples of this, the works of Piette (e.g., 29, 36) are recommended. A complete review of the subject has been given by Rich and Davidson (53).

Clearly, paramagnetic ions would be relatively useless for determination of structure of natural products if for no other reason than the fact that one only has relatively few paramagnetic ions to work with. With free radicals the compounds available are usually limited by the ingenuity (or lack of it) of the organic chemist.

However, one would now question whether one might use EPR as a technique to measure binding of paramagnetic ions. In general this is very difficult. The fact that most paramagnetic ions show their multipeaked spectra due to hyperfine interaction between a nucleus with an I value greater than one-half and an unpaired d electron indicates that it would be very difficult to disturb this environment. However, manganese with an I value of $5/2$ and an S value of $5/2$ shows both fine and hyperfine interactions. The theory of why it is much easier to disturb the normal relationship between the electrons and nucleus of manganese is very complex. Thorough discussions of the theoretical difficulties involving manganese are given in the works of Abragam (1, 19) and Gabriel (22). Hence for the special case of the ion manganese, EPR could be a useful technique in measuring binding of the ion.

As we previously mentioned, Leigh (38) showed that manganese in different conditions could show a decrease in amplitude without observable line broadening. As will be discussed in the results section of this dissertation, it was found that progressive dilution of a standard solution of manganese and measurement of average amplitude of the six peaks (i.e., sum of the amplitudes of the six peaks divided by six) showed a one-to-one correlation between average amplitude and concentration.

However, in considering binding studies, there is one very important point to be kept in mind. Unless one is dealing with a single known binder and can separate bound from unbound manganese, there is no way of telling what percentage of the ions is bound. It would only be possible if one were certain that all bound ions completely lost the spectra they had while unbound. However, this does not negate use of the technique for studying relative amounts of manganese bound. In a biological system such as is discussed in this dissertation, the eventual isolation of the binder and separation of bound and unbound manganese would permit answering the above question.

MATERIALS AND METHODS

Definitions and Abbreviations Employed

Definitions-

spontaneous

carcinogenesis.....The development of neoplasia by natural causes as contrasted to induction in the laboratory by known carcinogens.

induced

carcinogenesis.....The intentional development of neoplasia by known carcinogenic agents.

manganese binding

potential.....The ability of a given tissue to bind a known quantity of exogenous manganese per unit of protein in the tissue.

manganese.....Always refers to the divalent manganous ion.

endogenous

manganese.....The quantity of the manganous ion found in a given tissue sample.

exogenous

manganese.....The quantity of divalent manganous ion (in the form of manganous chloride) added to a given tissue sample.

All other terms employed have the same definition as utilized in current biochemical and biophysical literature.

Abbreviations-

Mn.....The divalent manganous ion.
MnCl₂.....Divalent manganous chloride tetrahydrate.
pro.....Units of protein (defined for each specific case).
MCA.....20-methylcholanthrene
DBP.....3,4,9,10 Dibenzyrene
DMBA.....9.10 dimethyl-1,2 benzanthracene
BPA.....Bovine Plasma Albumin

All other abbreviations and formulae utilized are those accepted in current chemical and biochemical literature, or are stated in the specific section where used.

Materials and Special Preparations

Materials-

All chemicals, unless otherwise specified, are reagent grade or of the highest purity commercially available. Manganous chloride, calcium chloride, nickel chloride and hydrochloric acid were products of Baker Chemical Company. Sodium chloride, sodium hydroxide and acetone were produced by Matheson, Coleman and Bell, Magnesium chloride by Merck Chemical Company and benzene, potassium sodium tartrate, copper sulfate and cobalt chloride by Malinckrodt. Hemoglobin was supplied by California Biochemical Corporation and BPA from various sources was utilized.

Peanut Oil of the supermarket grade was supplied by the Planters Peanut Company. All remaining carcinogens, metabolites, solvents, etc. were of the highest purity available from Sigma Chemical Company. All mice and tumors (with the exception of Swiss which are bred by PBRC animal colony at the University of Hawaii) were obtained from the Jackson Laboratory.

Special Preparations-

Biuret reagent was prepared by the procedure of Gornal et al (22) utilizing cupric sulfate, sodium hydroxide and sodium potassium tartrate. All saline solutions were physiological for the mouse (0.85%) and were prepared with glass distilled water as were all other preparations. All homogenizations were carried out in Ten Broeck homogenization tubes supplied by Van Waters and Rogers.

DBP suspensions utilized for intravenous injections were prepared by a method similar to that described by Andervont (6). The carcinogen was dissolved in a mixture of 18 ml acetone and 1 ml dimethyl sulfoxide. Water was then added such that the final concentration of DBP was approximately 2.4 mg/ml solution.

All other special precautions are described under the appropriate method in this section.

Instrumentation

All electron paramagnetic studies were carried out on a Varian Model E-4 Spectrometer.

A Perkin-Elmer Model 202 UV-Visible was utilized for all spectrophotometric readings taken.

A Sorvall Model RC-2B refrigerated centrifuge and a Beckman LC-65B ultracentrifuge were employed in studies requiring centrifugation.

Ultrafiltration was performed with a Model 52-65 milliliter ultrafiltration cell produced by Amicon Corporation.

All other equipment used (e.g., water bath, pH meter, etc.) was of the quality normally found in a well-equipped biochemistry laboratory.

Manganese Binding Assay System

The following is the technique used to measure manganese binding potential in tissue for the studies described herein.

1. An animal is sacrificed by etherization of cervical dislocation. The first technique was used on all studies except those involving brain and neuroblastoma.
2. Approximately 400 milligrams of the desired tissue is immediately excised, weighed and homogenized with 7 ml of 0.85% saline in a Ten Broecke glass homogenizer. In cases where a given organ weighed less than this,

a smaller amount was taken. However, in such cases the same approximate weight to volume of tissue to saline was taken on all studies performed of that organ type. All homogenizations are carried out in ice with much care taken that little heat was generated. In as much as it is possible, the time of homogenization and number of strokes is kept constant.

3. The suspension is then centrifuged at approximately 37,000 x g for 10 minutes in a refrigerated centrifuge at a temperature of 0° to 5° centigrade.
4. The supernatant is removed and 3-1 ml aliquots are taken.
5. 1.0 ml of 0.006 molar manganese standard (i.e., MnCl_2) is added to one aliquot (A).
6. The precipitate from the centrifugation in step 3 is homogenized with 4 ml of 0.85% saline and 3-1 ml aliquots are taken.
7. One aliquot from step 6 (B) is treated as in step 5.
8. The ESR manganese spectra of aliquots A, B and the Mn standard are determined. Proper positioning of the cell and frequent checks for reproducibility of the standard are essential.
9. 3 ml of biuret reagent are added to one aliquot from step 4 (C) and step 6 (D).
10. 3 ml of 3% sodium hydroxide are then added to the

remaining aliquots from steps 4 (E) and 6 (F). (This represents correction for heme absorbance.)

11. Aliquots C, D, E, and F are allowed to stand 30 to 45 minutes.
12. Aliquots C and E are filtered through a millipore apparatus utilizing vacuum suction and a HAWP 25 filter in each case.
13. Aliquots D and F are centrifuged at 37,000 x g for 15 minutes at 0°-5°C. (Centrifuge should be run with brake in the off position.)
14. The supernatants from step 13 are removed, CAREFULLY, and filtered through a millipore apparatus as in step 12.
15. The optical densities of aliquots C, D, E, and F are determined at 550 millimicrons with the appropriate blanks.
16. The manganese binding potential for the supernatant is the average height of the six peaks (sum of the height of all six peaks of the spectrum of the Mn standard minus the height of the average of the six peaks of aliquot A divided by 6) in centimeters over the total of (optical density of C minus optical density of E)/2.
17. In likewise manner, the manganese binding potential for the precipitate is the average height of the six peaks of the spectrum of the manganese standard minus that of

aliquot B in centimeters over the total

(optical density of D minus optical density of F)/2.

If one wished to determine approximate amount of manganese bound per milligram of protein, one could simply make calibration curves of the average height of the peaks for known concentrations of manganese and optical densities of biuret for known concentrations of protein and determine a conversion factor. However, the caution discussed previously (page 27) in interpreting the end result should be noted.

If the proper precautions are observed, one should be able to get reproducibility of manganese binding potential in a given organ type in a given strain of mice raised under similar conditions of ± 5% or less.

Heat Denaturation Studies

Since this is the primary evidence proposed in this dissertation for existence of the proposed inhibitor, it was felt worthwhile to outline the procedure as accurately as possible and to again re-emphasize several important cautions.

Steps 1-6

These are the same as steps 1-4 and 6 of the Mn binding assay system. NOTE: Manganese is not added until after the heating step. One again, importance of gentle homogenization in ice with as little generation of heat

as possible cannot be overstated.

Step 7

The fractions from the supernatant is placed in a water bath of the desired temperature for the desired period of time. Timing should be accurate, although second precision is not necessary.

Step 8

The tubes are removed and placed in ice for three minutes.

Steps 9-10

Steps 5 and 7 of manganese binding assay.

Techniques for Purification and Separation of Manganese Binding Components

The following is the technique utilized to elucidate something of the nature of the components of the manganese binding sub-system.

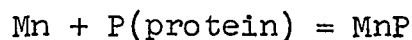
1. Step 1 of manganese binding assay system.
2. As much tissue as possible from a given organ is obtained. In the case of organs which have relatively small amounts of tissue, more than one animal may need to be sacrificed. The tissue is excised, blotted of excess blood, weighed and homogenized with 0.85% saline such that the final concentration is approximately 250 mg tissue per ml saline. In this work, at least one gram of tissue was utilized.

3. Step 3 of manganese binding assay system.
4. Step 4 of manganese binding assay system. These three aliquots are assayed for manganese binding, protein and heme as described in the Mn binding assay system.
5. The remainder of the supernatant is heated in boiling water for ten minutes.
6. Repeat steps 3 and 4 above. NOTE: At this time and for all future assays during this procedure, an aliquot need not be taken for heme as the concentration is now negligible.
7. Place the remainder of the supernatant in an amicon ultrafiltration cell with a UM05 filter. Add water to make the final volume approximately 45-50 ml and filter until approximately 10-15 ml remains. The procedure is repeated twice more.
8. Remove the remainder and wash the apparatus twice with 5-7 ml distilled water. Combine the wash with the remainder.
9. Assay both filtrate and remainder in step 8 as described in step 6. NOTE: No centrifugation is required.
10. Repeat step 7 using a PM-10 filter. NOTE: The procedure is repeated twice. However, the second and third filtrates are not combined with the first and can be discarded. DO NOT discard the first filtrate

as this contains the low molecular weight component of the manganese binding system and its loss makes future purification impossible. This will be referred as fraction R.

11. Repeat steps 8 and 9. Also assay for manganese binding by combining 0.5 ml of fraction R with 0.5 ml of the remainder and to this mixture add 1.0 ml of the manganese standard.
12. Future filtrations are accomplished using the desired filter and repeating steps 7 and 11. NOTE: Due to the fact that with filters of a higher pore size than the PM-10 the binder will pass through in varying amounts (depending upon the filter), one should assay each fraction of the filtrate before combining. This will also determine the number of times a filtration with a given size filter is run. (For example, the XM-50 filter appears to retain none of the binder. Clearly, the most useful purification here is to run the entire contents through the apparatus (i.e., no liquid remainder) as this will separate the binding agent from many higher molecular weight components.)
13. The Sephadex G-15 column bed volume was 22 cc. (The column height was 24 cm.) The flow rate was approximately 7 ml per hour.

If it is assumed that a bound manganese ion will not show an ESR signal, a hypothetical binding constant can be formulated. Assuming that the reaction is:



Then:

$$K_b = (\text{MnP})/(\text{Mn})(\text{P})$$

Hence:

$$K_b(\text{Mn}) = (\text{MnP})/(\text{P})$$

The term $K_b(\text{Mn})$ rather than simply K_b is used as the former gives a relative measure of purity.

Since (P) supposedly refers to the binding peptide and in most of the studies conducted the protein measured is total protein, the validity of $K_b(\text{Mn})$ might be questioned. However, it was shown that there appears to be a consistent ratio in different tissues between total protein of the initial homogenate and the binding peptide. As such $K_b(\text{Mn})$ still remains a useful term (relative) for comparisons.

As will be shown later, the binding system consists of a cofactor as well as the peptide binder. However, the fact of the former being greatly in excess justifies not adding it to the equilibrium expression.

For the relative loss of binder occurring during any purification procedure, one can calculate total binding as $(\text{MnP})(\text{total no. ml from purification})$.

Techniques of Trocaring and Carcinogenesis Experiments

Trocaring

At the appropriate time after implantation suggested by the Jackson manual (24), a tumor-bearing mouse is sacrificed by etherization. The tumor is excised after removal of the necrotic areas and the viable malignant tissue placed in a beaker of sterile 0.85% saline.

Immediately thereafter, small pieces of the tumor are placed in number 18 gauge trocar needles and trocared subcutaneously into (or as near to as possible to) the axillary region of the right front leg. The needle is gently removed, the skin is placed down and the animal is returned to its cage.

Notes, Cautions and Explanations

For each animal trocared, at least two pieces of tumor of approximate dimensions of 4 x 2 millimeters were always used in order to insure "a take". Also, at least three animals should be trocared so as to minimize the chance of losing the tumor.

It should be noted that from time to time, for various unknown reasons, a tumor will change its characteristics. Sometimes these changes are permanent; while at others, they are only temporary. These changes can be detected by such factors as a) approximate size of tumor at the time of death of the animal, b) approximate lifetime of an

implanted animal, and c) macroscopic appearance of the tumor at the time of death of the animal. Should a change in any of these parameters be noted, all experiments with a particular strain of tumor should be discontinued until the parameter returns to what is normally observed. If the tumor has undergone a permanent change, it should not be used for further experimentation (unless it is considered separately as a new tumor type). For further experimentation on the original tumor, the supplier should be contacted for newly implanted animals.

As much necrotic material as possible was removed before placing the tissue in saline so as to minimize chances of bacterial contamination.

Carcinogenesis Techniques

Intraperitoneal Injection

The carcinogen is suspended in peanut oil such that the desired amount can be injected in 0.1 ml of liquid. The suspension is injected using a 25 gauge needle. Care is taken to vigorously shake the syringe just prior to injection so as to get as uniform distribution of carcinogen as is possible. Clearly, there will be some variation in the dose received by each animal. Although a "necessary evil", the possible variation in results caused by this technique is covered in the Results and Discussion section of this dissertation.

Intravenous Injection

A detailed description of the method of preparing the carcinogen solution was given on page 30. Because of the very fine suspension resulting from this technique, a 27 gauge needle can be used for injections. However, as with the intraperitoneal injection technique, some variation in dose is to be expected.

Myeloid Leukemia Implantation

A small incision is made in the middle abdomen. With a sharp forceps, "a pocket" is made in one lobe of the liver. A freshly dissected piece of myeloid leukemia tissue is placed deep in the pocket. The mouse is then sutured to close the wound.

Other Procedures

Determination of Endogenous Bound and Unbound Manganese

It was found that addition of an equal volume of 1 normal hydrochloric acid to a tissue suspension to which manganese had been added would cause complete regeneration of the ESR signal of the manganese standard. This could then naturally be employed in a procedure to determine the amounts of endogenous bound and unbound manganese in tissues.

A given tissue is treated as is outlined in steps 1-4 and 6 of the manganese binding assay. No manganese is added and one aliquot of the supernatant and precipitate

are assayed for endogenous manganese on the ESR. 0.5 ml of each aliquot is then treated with 0.5 ml of 1 normal hydrochloric acid and the resultant solution assayed on the ESR. The first value obtained (before addition of HCl) should be an indication of approximate amount of unbound manganese. The latter value should indicate total endogenous manganese content. Hence a relative value for bound and unbound manganese can be obtained.

RESULTS, DISCUSSION, AND CONCLUSIONS

Progressive Manganese Dilution Experiments

Figure 2 shows a plot of average ESR signal size of a solution of 0.006 molar manganous chloride and various dilutions versus the concentration after dilution.

It is apparent that average signal size is a linear function of the dilution factor. This dilution experiment was performed in triplicate and this fact was established in all cases.

The above indicates that ESR can be used to measure manganese binding, providing binding does not change the T_1 and T_2 values, and hence increase in binding is an inverse function of amplitude.

To ascertain the validity of the above statement, spectra of appropriately diluted solutions of manganous chloride were compared with spectra obtained from solutions treated by tissue extracts. In all cases, the spectra were superimposable.

Manganese Binding Potential in Neoplastic and Non-Neoplastic Tissue

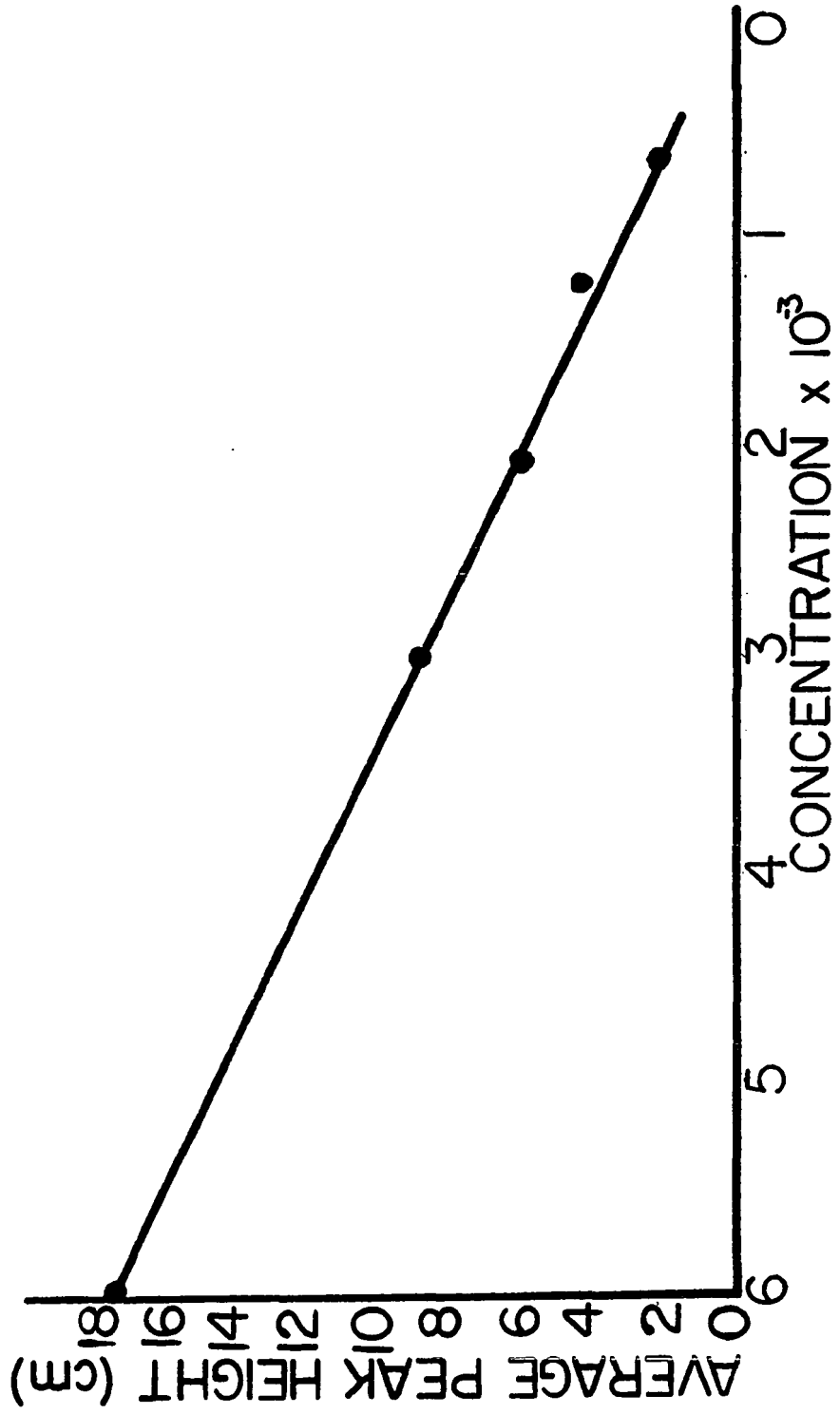
Tissues of the following tumors supplied by the Jackson Laboratory of Bar Harbor, Maine, and a carcinogen-induced tumor formed by implanting a crystal of 20-methylcholanthrene in the livers of strain C57Bl/6J mice

FIGURE 2

Progressive Manganese Dilution

A solution of 0.006 Molar Manganous Chloride was progressively diluted with distilled water.

Concentration is expressed in terms of final molarity $\times 10^{-3}$ after dilution. (For example, the number 3 represents a final concentration of 0.003 molar after a 1:1 dilution.)



were tested by the manganese binding assay system previously described (pages 31-34). The host tissue listed below was also tested.

1. Lymphatic Leukemia No. BW5147 transplanted in AKR/J mouse. Host Tissue: Spleen
2. Hepatoma No. BW7756 transplanted in C57L/J mouse. Host Tissue: Liver
3. Neuroblastoma No. C1300 transplanted in A/J mouse. Host Tissue: Brain
4. Myeloid Leukemia No. C1498 transplanted in C57Bl/6J mouse. Host Tissue: Liver

In all experiments involving tumor tissue, no necrotic tissue was taken. Samples were taken from the most highly vascularized area of the tumor with outer areas being taken first. The tumors were always tested within three days of the suggested time of re-implantation as stated in the manual issued by the Jackson Laboratory (24). All mice used, both with and without neoplastic disease, were of approximately 3 months of age and weighed approximately 25 grams. Only females were employed.

The results are shown on Table I. The number of runs refers to the number of different animals tested. Each experiment in a given system was run at different times to insure against machine-centered errors. In most cases the tumor and non-tumor animals in a given system were run

TABLE I

1. A complete description of each tumor system is given on page 46.
For further information, refer to the Jackson Manual (24).
2. Units of manganese binding potential are discussed on page 49.
3. Under the heading type, T refers to the Mn binding potential of the tumor while H refers to that of the host or tissue of origin.
4. Sol. and Insol. refer to aliquots A and B respectively as obtained from performance of the manganese binding assay system (page 31).
5. Total under the heading of manganese binding potential refers to the sum of the soluble and insoluble potentials.
6. Binding difference refers to the percentage drop in manganese binding potential of the tumor from that of the tissue of origin.

TABLE I

MANGANESE BINDING POTENTIAL IN NEOPLASTIC AND NON-NEOPLASTIC TISSUE

Tumor Type	No. Runs	Manganese Binding Potential				Binding Differences		
		<u>Type</u>	<u>Sol.</u>	<u>Insol.</u>	<u>Total</u>	<u>Sol.</u>	<u>Insol.</u>	<u>Total</u>
Lymphatic Leukemia	3	T	4.98	2.17	6.99	---	---	---
	3	H	9.13	3.86	12.99	45	44	46
Hepatoma	5	T	8.14	6.95	15.09	---	---	---
	3	H	14.50	10.07	24.58	44	31	39
Neuroblastoma	3	T	6.94	6.95	13.87	---	---	---
	3	H	8.01	15.77	23.78	13	56	42
20-Methylcholanthrene Induced Hepatoma	2	T	6.96	4.76	11.73	---	---	---
	2	H	9.01	7.19	16.20	22	51	38
Myeloid Leukemia	3	T	5.10	5.20	10.35	---	---	---
	2	H	9.01	7.19	16.20	43	27	32

during the same period to insure an accurate comparison. The numbers shown are averages of all the runs on a given tissue. In no instance was the total deviation greater than $\pm 5\%$ from the mean. The numbers on the table represent (average amplitude of 0.006 molar standard in cm minus average amplitude of dilution corrected tissue treated standard) divided by (OD biuret minus OD sodium hydroxide) times 2. The reason for doubling the denominator is that in certain instances (e.g., spleen) when only a small amount of tissue was available, the amount of biuret and sodium hydroxide added to the tissue sample was cut from 3 ml to 1 ml (total volume 2 ml instead of 4 ml) so as to obtain higher optical density readings and hence decrease the errors so inherent in low optical density readings. In all instances when this revised procedure was employed (only with AKR spleen) pH of the resulting tissue biuret and NaOH mixtures were carefully checked. No difference in pH was observed between this and samples treated by the procedure outlined in the manganese binding assay system (pages 31-34). To obtain the hypothetical $K_p(\text{Mn})$ in μg manganous ion bound/ μg total protein, one would multiply the numbers shown on Table I by the conversion factor 5.4×10^{-4} . The reason for expressing the values on Table I in the terms defined on page 46 rather than the more generally accepted term

stated above is that it is intended that these terms to be used as relative values when measuring manganese binding potential of such crude extracts. Even expressing these terms in mg Mn bound/mg protein might suggest certain incorrect interpretations. The fact that one has many contaminating proteins in these crude extracts would make any interpretation of a given value at best questionable and at worst completely incorrect. In binder purification studies use of the terms expressed in μg Mn bound/mg protein is justified so as to approximate number of amino acids present for each Mn bound and hence rule out the effect being due to an artifact. However, in all other instances, it is preferable to stay as far as possible from this "necessary evil". The conversion factor is included above mainly for completeness.

It can be seen from Table I that in all instances, the tumor tissue has less manganese binding potential than does its tissue of origin. A justifiable criticism of these results would be to point out that other proteins that can bind manganese. This is quite true. However, aside from several compounds that are present in very minor quantities in the cell, the major binding effect comes from phosphate compounds. And as Greenstein (25) points out, it appears (as would be teleologically expected from the fact that malignant cells are rapidly proliferating) that

they possess greater amounts of these compounds than their respective host tissues. Hence, were this the determining factor, one would expect malignant tissue to be more able to bind manganese than their tissues of origin. It has been found that the opposite is true.

Clearly, it is necessary to point out that in order to make a conclusive definition in experiments of this type, one would have to have a complete biochemical profile on both the neoplastic and non-neoplastic tissues under consideration. However, it was felt that the data from these experiments, although not conclusive, were indicative of a difference between malignant tissue and tissue of origin. And although a complete biochemical profile would be ideal, this has not yet been accomplished for any tissue. Hence it was felt an adequate compromise would be to proceed to attempt purification of the binding agent(s) and to determine whether manganese binding potential changes during the carcinogenic process. The results of these and other experiments are presented on the following pages.

Ion Competition Studies

These studies were conducted in order to gain evidence on the specificity of the binder. The fact that it is difficult to separate the bound manganese from the binding agent(s) indicates a relatively strong binding constant.

If the binder(s) are relatively non-specific one would expect fairly strong binding constants for certainly some of the other ions that resemble manganese either in electropositivity, size or both. Hence, addition of these ions in concentrations much in excess of concentrations of manganese added should prevent manganese from being bound and hence the ESR signal of the manganese in the presence of the ion should be greater than that of manganese alone. For these experiments, we took equal aliquots of homogenates of Swiss mouse livers as prepared by the procedure described in the manganese assay system (page 32). In the first series of experiments, solutions of MnCl_2 (0.006 M) containing no other ions or either chloride salts of Mg, Ca, Fe, Co, or Ni (with the MnCl_2) in concentrations of 0.6 molar (i.e., 100 x concentration of MnCl_2) were added to tissue aliquots as described in the manganese binding assay. The average height of the 6 Mn peaks was calculated in each case. In the case of ions that show paramagnetism at room temperature (e.g., Cu), a blank was run of the ion plus tissue with no Mn added. The results of these experiments are shown in Table II. In a second series of experiments, the salt solution of the ion being tested for competition is added to the tissue aliquot prior to the addition of the manganese standard. The blank is addition of glass distilled water in lieu of a salt solution. The

TABLES II AND III

1. S and P refer to aliquots A and B respectively as obtained from performance of the manganese binding assay system (page 31).
2. Cu was the only ion tested that gave an ESR spectrum in the same range as manganese. Although the results appear to indicate little competition it is necessary to point out that subtraction of the Cu blank is a crude procedure at best.
3. The Zn concentration was somewhat less than 0.6 molar. This is evidenced by formation of a clouded suspension in the zinc chloride solution. The excess of Zn as compared to manganese was not determined.
4. % difference refers to the difference between the average peak height of the manganese standard alone and that solution with the competing ion present.

TABLE II

COMPETITIVE BINDING STUDIES
IONS ADDED SIMULTANEOUSLY

Competing Ion	Fraction	Average Size of Mn Signal	% Difference
None	S	15.6	---
None	P	16.4	---
Mg	S	15.0	-4
Mg	P	15.6	-5
Ca	S	15.0	-4
Ca	P	16.0	-2
Co	S	14.0	-10
Co	P	14.2	-14
Ni	S	14.8	-5
Ni	P	15.2	-7
Cu	S	15.0	-4
Cu	P	15.4	-6
Zn	S	15.0	-4
Zn	P	15.2	-7

TABLE III

COMPETITIVE BINDING STUDIES
COMPETITIVE ION ADDED BEFORE MANGANESE

Competing Ion	Fraction	Average Size of Mn Signal	% Difference
None	S	15.2	---
None	P	15.6	---
Mg	S	14.4	-5
Mg	P	15.8	+1
Ca	S	15.0	-1
Ca	P	15.4	-1
Co	S	13.8	-9
Co	P	14.0	-11
Ni	S	14.6	-4
Ni	P	14.8	-5
Cu	S	14.8	-3
Cu	P	15.0	-4
Zn	S	14.4	-5
Zn	P	15.0	-4

results of these experiments is shown in Table III. The order of the elements of both tables is not arbitrary, but in order of increasing electropositivity.

It can be seen in both experiments that with the exception of cobalt there is little difference in size of the manganese signal with or without the presence of the other ion. The presence of cobalt causes a decrease in signal size and hence suggests increased manganese binding. This is an interesting observation and should be looked into in more detail. However, in no instance can the ion added with manganese be said to be competing.

The above experiments were repeated with similar results. It is thus felt that the above experiments add evidence to the thesis of the observed binding of manganese being of a specific rather than of a general nature.

Endogenous Bound and Unbound Manganese in Neoplastic and Non-Neoplastic Tissue

The procedure followed is outlined on pages 41-42. The results as obtained in three of the systems is shown in Table IV. These numbers represent average height of the peaks over OD 550 biuret minus OD 550 sodium hydroxide. It is suggested that the variation in results from a given tissue in different animals is most likely due to the inaccuracy obtained from the low signal to noise ratio that accompanies ESR spectra run at such high gains. The studies

TABLE IV

1. The three tumor-tissue of origin systems are numbers 2, 3, and 4 described on page 46.
2. Precipitate and supernatant refer to aliquots obtained from steps 6 and 4 respectively of the modified manganese binding assay system (pages 41-42).
3. The total signal simply refers to the sum of the signals from the supernatant and precipitate.
4. The conversion factor for these numbers based on the difference in gain from exogenous manganese experiments for $K_b(\text{Mn})$ is 1.08×10^{-6} . Certainly in this experiment care must be taken in interpreting $K_b(\text{Mn})$ in any more than a relative number due to errors coming from the high signal to noise ratio of the instrument as well as the other points mentioned throughout the dissertation.
5. Approx. % Unbound Mn for a given fraction is (signal without HCl)/(signal with HCl). In a very superficial sense this might be considered a rough estimate of unbound endogenous manganese (relative).

TABLE IV

ENDOGENOUS BOUND AND UNBOUND MANGANESE IN NEOPLASTIC AND NON-NEOPLASTIC TISSUE

Tissue	Precipitate		Supernatant		Total Signal		Approx % Precip.	% Unbound Mn	
	-HCl	HCl	-HCl	HCl	-HCl	HCl		Super	Total
Brain	neg	3.54	5.5	19.0	5.5	22.54	---	29	24
Neuroblastoma	neg	neg	10.6	17.0	10.6	17.0	---	62	62
Brain	neg	2.31	4.4	22.0	4.4	24.31	---	20	18
Neuroblastoma	neg	neg	11.5	17.0	11.5	17.0	---	68	68
58 Liver--C57L/J	1.49	2.81	3.11	30.2	4.60	33.01	53	10	14
Hepatoma	1.71	2.41	11.21	32.7	12.92	35.11	71	34	39
Liver--C57L/J	1.26	3.41	2.00	29.8	3.26	33.21	37	7	10
Hepatoma	1.89	2.20	9.34	36.7	11.23	38.9	86	25	29
Liver--C57Bl/6J	1.11	2.39	11.2	42.5	12.31	44.89	46	27	27
Myeloid Leukemia	1.97	2.42	29.1	43.6	31.07	46.02	81	67	68
Liver--C57Bl/6J	1.00	2.51	10.2	42.2	11.2	44.71	40	24	25
Myeloid Leukemia	2.25	2.64	25.6	42.1	27.85	44.74	85	61	62

involving addition of exogenous manganese are run at much lower gains and hence one does not experience this problem.

This mechanical problem points out what is perhaps the most difficult and teleologically disturbing point of the work. The approximate amount of manganese binding observed upon addition of exogenous manganese is 200-500 times greater than the amount of manganese normally found in the cell. It is incomprehensible why this is the case. One can postulate many mechanisms to explain how this occurs. One could technically divide these theories into two classes.

The first class would infer that somewhere between the time the animal was killed and the time the exogenous manganese was added a change occurred that caused greatly increased binding ability. The number of sub-theories that could be constructed to explain exactly what happened is great. As an example, since it has been shown that the binding system needs two components (purification experiments), one might say that the two compounds are fairly well compartmentalized and that upon homogenization, the agents mix and hence increase binding. Another alternative would be to suggest that the postulated inhibitor was present in very large quantities. Since it is quite labile, the homogenization, no matter how gentle, destroys much of it and hence increased binding is observed. Clearly, these are simply speculation.

The second class would make no speculation as to changes involved in the physical processes of the experiment. Rather it would postulate that the endogenous manganese were in some way prevented from binding. Physical homogenization would in no way alter this state (i.e., of the endogenous manganese) although it might change the state of the binding or inhibiting systems. Sub-theories would diverge from here.

Logic dictates that the second class of theories be accepted. It is known that addition of hydrochloric acid causes regeneration of the ESR signal and release of the bound manganese probably by destruction of one of the binding components. (Subsequent return of HCl treated solutions to pH of 7 showed no loss of the regenerated signal aside from that caused by dilution.) It therefore follows that any tissue extracts showing the same size peaks per given amount of protein after HCl addition would have the same total amount of manganese. Thus, unless one postulates more than one binder, one would expect tissues with equal amounts of total manganese to give the same size signals prior to addition of the hydrochloric acid. It can be seen from Table IV that this is not so. In each system tested, the tumor tissue shows a greater signal than its tissue of origin prior to addition of HCl. This would suggest that tumor tissue had more unbound endogenous

manganese than its tissue of origin. This would be expected on the basis of the theory.

One theory that was postulated was that endogenous manganese in the soluble fraction was prevented from binding due to its localization in the ribosomes. (Most mitochondria, nuclei and cellular material would be found in the precipitate fraction after centrifugation at 37,000 x g.) To test this, endogenous manganese levels in the soluble fraction of C57L/J liver were measured before and after centrifugation at approximately 115,000 x g for 2.5 hours. The endogenous manganese found to be present in the supernatant from the 37,000 x g centrifugation was not found in the supernatant from the ultracentrifugation. However, resuspension of the ribosomes showed that the unbound ion was present in this fraction. It is thus suggested that for the soluble fraction at least, the reason that there is unbound manganese is due to its localization in the ribosomes preventing complete binding with the system. It should be pointed out that a complete profile of localization of endogenous manganese and the components of the binding system in the cell is necessary to settle this point. Work of this nature is currently being pursued.

HOWEVER, these experiments with endogenous manganese prove nothing. They can at best be considered a weak

indication to support the theory. The number of possible fallacies that could be presented to counter any interpretation is phenomenal. Before the entire system is characterized with each component isolated and identified, no interpretation can be made. It is hoped that the experiments planned will explain these results.

Purification of the Manganese Binding Sub-System

The general procedure employed is stated on pages 35-37. Sephadex molecular seive chromatography as will be discussed below was omitted in the initial experiments. Typical purifications involving Swiss mouse livers are shown in Tables V and VI. Other purifications involving C57L/J livers and hepatomas and Swiss normals and dibenzpyrene treated mice livers are shown in Tables VII-XII. It should be noted that in these last four purifications the last step was followed by Sephadex G-50 gel filtration. What appeared to be a good purification technique was in actuality the total material coming off in bed volume (i.e., the molecular weight of the components present is 1500 or lower) and gave no real purification. This was an error in judgment in interpretation of the ultrafiltration results and since nothing can be gained from this data, it is omitted. A purification of livers of normal Swiss and 3,4,9,10 dibenzpyrene treated livers was performed utilizing a Sephadex G-15 column filtration after the ultrafiltration

TABLES V TO XII

Abbreviations Used and Parameters of Expression

1. (MnP) refers to bound manganese. It is expressed in micrograms per milliliter. It is determined by measuring the difference between average peak height of 0.006 M manganese chloride standard and that of the solution treated with the extract from a given step.
2. (P) x 10³ refers to protein in micrograms per milliliter. As an example in Table VI, 25.80 represents 25.80 x 10³ micrograms/milliliter.
3. K_b(Mn) x 10⁻³ is the figure obtained by dividing (MnP) by (P). Thus in Table VI for the homogenization step, K_b(Mn) is 60/25.8 x 10³ or 2.15 x 10⁻³.
4. Total Ml. refers to the total number of milliliters of solution obtained from a given purification step. As such, Total (MnP) is simply the product of (MnP) and Total Ml.
5. % Rec. is the abbreviation for percentage recovery. It refers to the quotient of the total number of micrograms of manganese that could be bounded by the entire fraction from a given step and the amount that

could be bound by the total initial fraction from homogenization. The latter term is corrected in each step for that percentage of the initial fraction that was used for assay in all previous steps.

6. Homog. refers to the supernatant obtained from the initial homogenization.
7. Boil represents the supernatant fraction obtained after boiling as described on page 35. Boil P is the precipitate from this fraction. It is tested by homogenizing this precipitate in saline.
8. UM-05, PM-10, PM-30 and XM-50 are the designations given by Amicon Corporation to their ultrafiltration membranes.
9. The letters R and F following the term for a given filter (e.g., UM-05R) refer, respectively, to the remainder and filtrate obtained by use of the given filter.
10. The letter C when used with PM-10 refers to the results obtained when an equal aliquot of the remainder and filtrate from filtration with a PM-10 membrane were combined. The letter C when used with another filter number and an R or F refers to testing of a combination of the remainder or filtrate, respectively, from that filter with an equal

volume of the filtrate obtained from the PM-10 filtration (i.e., the low molecular weight cofactor).

11. DBP refers to the carcinogen 3,4,9,10 dibenzpyrene. In the experiment referred to by Table XI, 1 mg of the compound suspended in 0.1 ml peanut oil was given intraperitoneally 52 days earlier. In the experiment described in Table XII, the compound was given 72 days earlier. All animals tested were distended with ascitic fluid which upon microscopic examination was found to contain vast numbers of malignant cells.

12. The number found in the title of certain tables simply refers to the particular given experiment with a given type of mouse. (For example, the (3) in Table X simply means that this was the third experiment performed on normal Swiss mice.)

TABLE V

PURIFICATION EXPERIMENT--SWISS LIVER (1)

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_pMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	60	25.80	2.15	33	1.98x10 ³	---
Boil	41	7.35	5.67	29.5	1.21x10 ³	67
UM-05R	26.4	5.82	4.54	36	0.95x10 ³	57
UM-05F	---	---	---	---	---	---
PM-10R	2.9	13.15	0.29	15	43.5	3
PM-10F	---	---	---	---	---	---

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TABLE VI

PURIFICATION EXPERIMENT--SWISS LIVER (2)

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_pMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	62	30	2.08	37	2.25x10 ³	---
Boil	43	7.42	5.8	35	1.51x10 ³	68
UM-05R	51	9.35	5.45	27	1.38x10 ³	64
UM-05F	---	---	---	---	---	---
PM-10F	---	---	---	---	---	---
PM-10R	3.0	15.8	0.19	15	0.045	2.1
PM-10C	36	7.9	4.56	30	1.08x10 ³	51

TABLE VII

PURIFICATION EXPERIMENT--C57L/J LIVER

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_pMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	148	25.56	5.63	52.5	7.63x10 ³	---
Boil	107.4	6.45	16.7	50	5.37x10 ³	77.5
Boil-P	26.7	N.C.	---	40	1.07x10 ³	15.2
UM-05R	119.7	6.80	17.6	40	4.78x10 ³	68.8
PM-10C	146.3	9.00	16.3	40	4.39x10 ³	66.3
PM-30RC	26.7	1.61	16.6	40	1.07x10 ³	17.5
PM-30FC	47.4	3.38	14.0	60	2.84x10 ³	45.7
XM-50RC	Neg.	1.29	---	25	---	00.0
XM-50FC	142	8.55	16.5	26	2.22x10 ³	59.0

TABLE VIII

PURIFICATION EXPERIMENT--C57L/J HEPATOMA

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_bMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	84	25.56	3.28	51	4.28x10 ³	---
Boil	75	6.48	11.58	50	3.75x10 ³	95
Boil-P	18	N.C.	---	44	0.80x10 ³	19.9
UM-05R	120	12.96	9.26	27.5	3.30x10 ³	86.3
PM-10C	87	13.61	6.40	22	1.92x10 ³	68
XM-50RC	72	13.94	5.18	18	1.30x10 ³	45.2

TABLE IX

PURIFICATION EXPERIMENT--SWISS LIVER (3)

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_pMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	55.5	25.80	2.15	32	1.77x10 ³	---
Boil	40.5	7.10	5.7	30	1.22x10 ³	72
UM-05R	49.8	10.22	5.87	22	1.10x10 ³	66
UM-05F	---	---	---	---	---	---
PM-10C	33	7.93	4.16	28	0.93x10 ³	62
XM-50C	33	9.51	3.47	18	0.60x10 ³	42

TABLE X

PURIFICATION EXPERIMENT--SWISS LIVER--DBP TREATED

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>(K_pMn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	36	23.22	1.55	28	1.01x10 ³	---
Boil	31	7.42	4.17	25.5	0.79x10 ³	87
UM-05R	28	7.25	3.86	25	0.70x10 ³	80
UM-05F	---	---	---	---	---	---
PM-10C	15.8	5.8	2.72	28	0.45x10 ³	54
XM-50C	15.5	6.45	2.40	24	0.37x10 ³	48

TABLE XI

PURIFICATION EXPERIMENT--SWISS LIVER (4)

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>K_b(Mn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	52	23.67	2.21	11.5	5.98x10 ²	---
Boil	34.2	5.80	5.90	10	3.42x10 ²	65.7
UM--05R	16.1	3.07	5.23	17	2.74x10 ²	59.4
UM--05F	---	---	---	---	---	---
PM--10C	9.65	1.94	4.97	26	2.50x10 ²	57.0
XM--50C	5.05	1.46	3.46	30	1.51x10 ³	40.6

TABLE XII

PURIFICATION EXPERIMENT--SWISS LIVER--DBP TREATED (2)

<u>Step</u>	<u>(MnP)</u>	<u>(P)x10³</u>	<u>K_p(Mn)x10⁻³</u>	<u>Total Ml</u>	<u>Total (MnP)</u>	<u>% Rec.</u>
Homog.	34.5	23.8	1.45	12	4.14x10 ²	---
Boil	33.0	8.06	4.23	10	3.30x10 ²	96.5
UM-05R	11.25	3.06	3.68	20	2.25x10 ²	78.5
PM-10	6.15	2.26	2.72	25	1.54x10 ²	55.5
XM-50	4.05	1.78	2.27	25	1.01x10 ²	39.1

steps. This proved to be an excellent means of purification and the results are shown in Tables XI and XII and Figures 3 and 4. The results will be discussed later in this section.

Parameters Utilized

Although the next few paragraphs are repetitious of what is contained in the notes on the various tables in this section, it is felt that their tremendous importance justifies this extravagance. The table shown for each purification represents each parameter in micrograms as is discussed below. From the data presented, one should easily be able to make any other calculations desired. It is felt that the data presented represents the information in its clearest form. It should be added that for each step in each purification (through ultrafiltration), an attempt was made to calculate maximum molecular weight of the binding agent. Assuming an average molecular weight of amino acid of 120, the lowest value obtained for this was 2,560. As will be seen later the molecular weight as calculated from the G-15 column is approximately 1400. It can be assumed that a fairly good purification was obtained from ultrafiltration.

An attempt will now be made to define each term as precisely as possible.

(MnP) refers to bound manganese and is expressed in micrograms per ml. The value is determined by calculating

FIGURE 3

Sephadex G-15 Filtration for Normal Swiss Mice

The figure shows purification of the binding agent in normal Swiss mouse livers on Sephadex G-15 after previous purification steps (see text). The bed volume was approximately 22 cc with a height of 24 cm.

(P) refers to protein concentration in micrograms/milliliter.

(For example, 4 would represent a concentration of 4×10^3 micrograms per milliliter or 4 milligrams/milliliter.)

(MnP) refers to micrograms of bound manganous ion per milliliter. (For example, 4 would represent a total of 4×5 or 20 micrograms of manganese bound/milliliter.)

Each aliquot as defined on the figure contained approximately 3 milliliters.

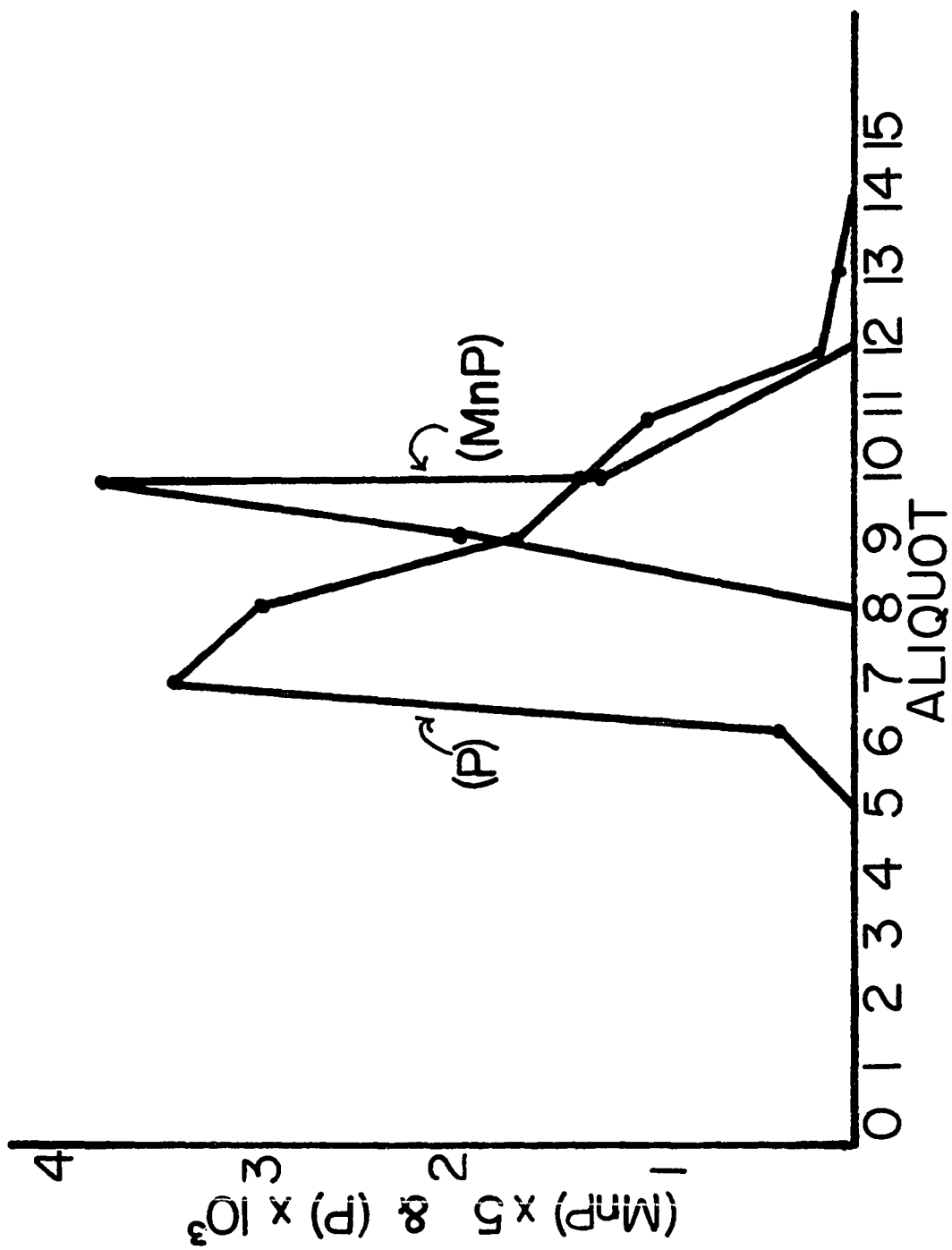
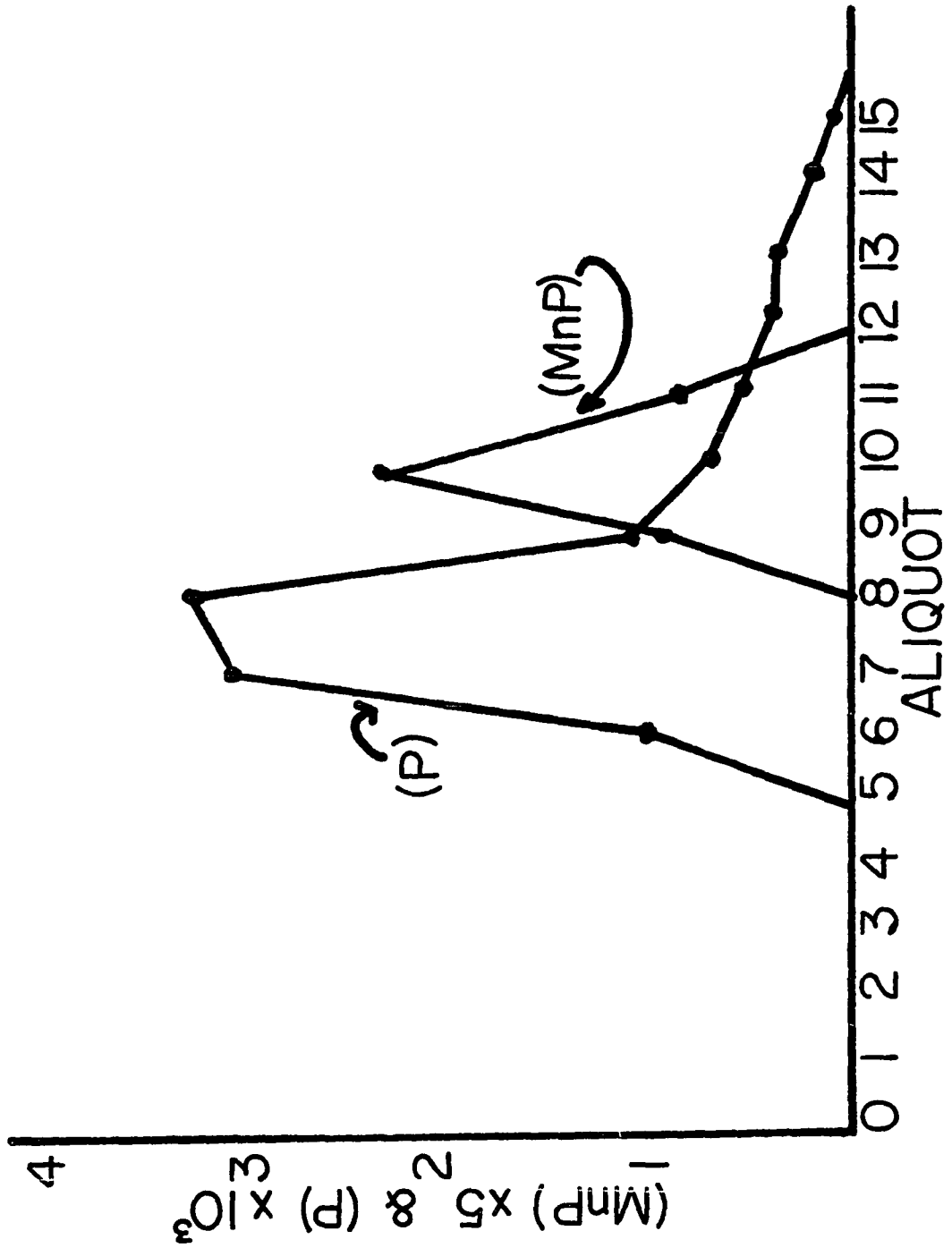


FIGURE 4

Sephadex G-15 Filtration for DBP Treated Swiss Mice

The figure shows purification of the binding agent in livers of Swiss mice given 1 mg of 3,4,9,10 dibenzpyrene in 0.1 ml peanut oil I.P. days before the experiment. The G-15 filtration was performed after previous purification steps (see text). Column dimensions and parameters utilized in the figure is the same as in figure 3.



the percentage of 0.006 molar manganous chloride standard bound by observing the average peak height before and after adding the respective tissue fraction.

(P) refers to total protein concentration in micrograms per milliliter.

$K_b(\text{Mn})$ is simply $(\text{MnP})/(\text{P})$ as is described by the equilibrium expression on page 38. Although technically the equilibrium is only correct if it is assumed P represents the combination of protein and cofactor, it appears from the results (see discussion below) and is logical to assume that the cofactor is in excess. Hence, the equilibrium would depend primarily on the protein and it is felt that the definition of $K_b(\text{Mn})$ is justified.

Percentage recovery refers to total (MnP) for a given step divided by total (MnP) of the initial homogenization with the amount used for assay in each step taken into account. (Corrections are made for aliquots used in testing.) As with any purification procedure, the percentage recovered is as important as the magnitude of purification.

The relative values of $K_b(\text{Mn})$ of course yield a criteria for purification somewhat like specific activity in enzyme work.

Great pains have been taken to make the results in this section as complete as possible, since elucidation of

the manganese binding system, as well as being the crux of this dissertation, is also the key point to any further exploration of this field.

An attempt will now be made to examine each step of the purification individually in order to ascertain as much as possible about the nature of the binding system.

Analysis of Results

The first step in the purification, after the initial homogenization, is the placing of the extract in boiling water for ten minutes. Examination of Tables V-XII shows a total loss in activity of various values between 22.5% and 33%. As can be seen (Table VII) rehomogenization of the boiled precipitate shows in the case of normal animals (Swiss) that the total amount of manganese bound by this fraction and the supernatant from boiling is within 10% of that bound by the initial homogenate. This would indicate a good deal of physical entrapment of the binding agent with little destruction of activity. As it will be shown later that the binding system consists both of a protein and cofactor, one must assume that the binding protein is either associated with the cofactor in the precipitate of the boiling step or that the cofactor is in such excess that enough is present in the "plug" to allow binding. Addition of excess cofactor (i.e., filtrate from the PM-10 ultrafiltration) to suspensions of boiled precipitate

caused no increase in the binding activity of this fraction. This would further substantiate the hypothesis that the cofactor was associated or in excess.

Whereas the total activity of the boiled fractions from normal animals was somewhat less than that of the initial homogenate, it can be seen that in animals with malignancy (Table VIII), the sum is substantially greater than this initial fraction. Indeed, whereas examination of the supernatants from boiling in the case of normal animals shows a distinct loss of activity (Tables V-VII, IX and XI) when compared to the initial fraction, in the case of animals with malignancies (Tables VIII, X and XII), the loss is of a much lesser magnitude. Whereas this latter point suggests little, the fact of the combined supernatant and precipitate boiled fractions showing greater activity than the initial homogenate is clearly difficult to explain unless one predicts the existence of an inhibitor of binding. It is felt that these results, as well as specific studies discussed later in this dissertation (pages 99-101) do indicate the existence of such an agent. As will be shown, if such an agent does exist, it is extremely labile.

Practically, as can be seen from all the tables, boiling (i.e., placing in boiling water for ten minutes) is an excellent purification technique. Although there is loss of activity, the value for $K_b(\text{Mn})$ increases substantially.

One may now summarize the findings from the boiling purification step.

- (1) The binding components of the system in the unpurified state in both malignant and non-malignant tissue are quite stable to heat.
- (2) There is some physical entrapment of the binding components in the precipitate. The cofactor is in excess or associated with the protein (see page 86).
- (3) The malignant tissue appears to possess some type of binding inhibitor which is destroyed upon heating. These purification studies do not indicate the agent to be present in non-malignant tissue tested.

The purpose of the filtration with the UM-05 membrane was primarily to rule out the possibility of the observations being due to simple charge effects. According to the brochure put out by Amicon Corporation, the percentage retention of sucrose (mw = 342) at pH 5 and 10 is 80% and 70% respectively. The retention properties of lower molecular weight components vary drastically with pH. Higher molecular weight components, with few exceptions, are almost completely retained. Thus, the UM-05 membrane could be expected to remove most ions present as well as phosphate, pyrophosphate and possibly some mononucleotides (e.g., AMP has a molecular weight of 347, quite close to

that of sucrose mentioned above). One would expect to see some loss in binding activity simply due to decrease in the amount of P_i , PP_i and mononucleotides. And in fact, in all experiments performed, a loss of activity although not of great magnitude was observed. There can be no true correlation made between loss of activity in malignant and non-malignant tissue. The losses for both types of tissue are similar and observation of the K_b (Mn) of these tissues before and after UM-05 filtration shows that after the procedure is employed the K_b (Mn) of the malignant tissue (or carcinogen treated) is still significantly lower than the normal tissue of origin. It should be noted that in all cases, the fraction was filtered through the UM-05 membrane at least two times. In many cases (Tables V, VI and VII), they were filtered three times. There appears to be no appreciable difference observed in those fractions filtered two or three times. It should be added that in previous work performed with the Amicon ultrafiltration apparatus, it was found that two runs is quite sufficient for removing the majority of ions. However, to be certain that charge effects were not responsible the three filtration experiments were run. This would effectively remove most if not all of the ions. One might now question as to why one shouldn't run experiments involving three filtrations just "to be on the safe side". The answer is that these

filtrations utilizing the UM-05 membrane are exceedingly slow. Since there is no appreciable difference in binding activity in those fractions filtered two or three times and the majority of ions are removed with two filtrations, the loss of time incurred by addition of the third filtration seems pointless. It may be thus concluded that filtration through the UM-05 membrane tells the following.

- (1) The binding of manganese that is observed is not a charge dependent process.
- (2) The differences in binding between malignant and non-malignant $K_b(\text{Mn})$ is not due to differences in phosphate or pyrophosphate content of the cells (although nothing as yet has been proven with regard to low molecular weight phosphate compounds).

One can now examine the step involving ultrafiltration through the PM-10 membrane. According to the Amicon brochure (62) all compounds tested with molecular weights less than or equal to that of raffinose (mw = 594) showed no retention in the cell. The compound of next higher molecular weight tested was bacitracin (mw = 1400) with 55% retained. A most important point should be made here. When the Amicon brochure refers to any percentage retention, it means that amount retained when the cell is flushed of all liquid. In practice, one rarely does this. The reasons

are twofold. (a) A good proportion of many of the compounds in the cell are trapped in the pores of the membrane. (We and other colleagues have learned this from sad experience.) So unless one is interested only in a compound that passes through the membrane (and here too if the molecular weight is near the pore size, a certain amount will be retained) one will lose a good proportion of the compound of interest by this entrapment. (b) The removal of liquid from many samples (primarily proteins) can cause denaturation. Thus when the brochure refers to a compound such as raffinose having 0% retention, in practice one rarely achieves this unless one flushes several times. As an example, if the cell is initially loaded with 50 mls of liquid and is brought down to 5, 10% of the raffinose will remain. Performing the same filtration with the same initial and final volumes again will reduce it to 1% of its initial concentration. A further filtration would cause 0.1% to remain, etc. One would suspect that when the brochure refers to a given amount of retention, there has been no attempt to measure the amount passed through the filter to determine whether the sum of the two figures is 100%. It should also be noted that the majority of compounds mentioned are fairly stable and hence not too likely to be denatured by loss of liquid. Thus from the data, one can determine little about the amount of entrapment or

denaturation obtained by use of a given membrane and various molecular weight compounds.

One other point should be mentioned. In the Amicon brochure, no mention is made of retention values for highly charged compounds. In our past experience using these membranes, highly charged compounds sometimes separate very strangely (i.e., not simply on the basis of molecular weight). Thus although in general these membranes will give a good approximation of molecular weight of a compound, there are exceptions.

The above section on physical limitations of the membranes was added for, although they have been used heavily in the purification procedure and are found to be an excellent tool, it is necessary to point out their drawbacks. However, it should again be reiterated that in the case of the desalting with UM-05 that was previously mentioned, from past experience it is known that this technique is quite valuable.

Evidence for a Low Molecular Weight Cofactor

In the initial purification experiment, it was with great consternation that it was learned that while the filtrate had no observable activity, the remainder only had 3% of the initial activity, while in the previous step 57% had been obtained (Tables V and VI). It was concluded that there was a possible synergistic effect between an agent in

the filtrate and one in the remainder. In this first experiment the filtrate fractions from the two-fold filtration with the PM-10 were combined. It should be stressed that in all other experiments mentioned, the filtrate fraction from the first PM-10 filtration is used as the source of the low molecular weight cofactor. It was shown in all subsequent experiments that a combination of both filtrate and remainder (Tables VI to XII) did cause regeneration of the majority of activity. Thus, there does appear to be two components required for the manganese binding to occur.

Examination of the filtrate from the PM-10 filtration showed no detectable protein. Ultraviolet spectra run on various samples showed a peak at 260 millimicrons indicating the solutions to contain any of the following: free nitrogenous base, nucleotides or nucleosides. The fact that these compounds are indicated to be present does not prove that any are the low molecular weight cofactor, per se. But it is felt that there is certainly a strong possibility of this being the case.

Although there is some binding in the remainder fraction alone which would be expected (as not all of the low molecular weight cofactor is removed by filtration), one might question why there is no binding observed in the filtrate. One might expect some binding for several

reasons. (a) One would expect some nucleotides to pass through the membrane and it is known that phosphate compounds do bind manganese. (b) Since it has been shown (page 94) that the molecular weight of the peptide component is approximately 1400, one would expect some of this compound might pass through the membrane.

The second explanation can be ruled out. One can boil the filtrate fraction to as low as 1/10 of its volume and be able to detect no protein present. Although a small amount of protein (too low for detection) might be present, it certainly is too small to have little consequence.

The first explanation is quite difficult to pin-point. One might suggest several reasons for lack of binding. (a) After dilution from the filtration, the binding values were too low to measure under the gains that were used. (Higher gains on the ESR were not tested.) (b) The phosphate compounds did not pass through the membrane in any substantial quantity. (c) The nucleotides do not offer an appreciable amount of binding. (d) A good percentage of these compounds could have been lost from the boiling (i.e., physical entrapment) or UM-05 steps. Any or all of these four explanations could be correct. However, it was felt that the long and tedious experiments necessary to explain this would not justify the results obtained. This is so because comparison of the $K_p(\text{Mn})$ of malignant and

non-malignant tissue after this filtration still shows that of the malignant tissue to be of a much smaller magnitude. This, it is felt, is the importance of the experiment. This filtration shows quite strongly that differences in binding between the two tissue types (malignant and non-malignant tissue of origin) is not solely due to tissue differences of low molecular weight phosphate compounds. Were this so, one would at least expect a split in binding between the remainder and filtrate as certainly a good proportion of mono- and diphosphates must pass through the membrane. The synergistic effect can only be explained by postulating a two component system.

But although it seems fairly evident that differences between neoplastic and non-neoplastic tissue are not due simply to low molecular phosphate differences, an important question to ask is which component (the low molecular weight cofactor or the protein) is in excess. Each calculation of Total Ml. was made by doubling the volume of the protein fraction as there was always a much greater volume of the low molecular cofactor and because the following experiments proved that it was the cofactor that was in excess on an equal volume basis. This data was not included on the tables as no quantitative measurements were made. Addition of excess cofactor component to protein fractions from either the PM-10, XM-50 or the Sephadex G-50

aliquots having activity did not yield any increase in binding. However, addition of excess protein fraction from any of these steps to a combination of protein and cofactor produced a definite increase in binding. This would certainly tend to indicate the component in excess is the cofactor.

Perhaps the most exciting experiment in these purification experiments is one where one takes a normal test aliquot from the XM-50 purification step of the C57L/J hepatoma to which an equal volume of the filtrate from the PM-10 had been added and adds excess XM-50 fraction from the normal C57L/J liver. A distinct binding increase occurred. This opens up all types of interesting suggestions on possible use of the protein binder as a chemotherapeutic agent. Of course, such a use presupposes many things such as permeability to cellular membranes. No experiments of this nature have yet been performed (although it is anticipated in the near future). But the suggestion itself is certainly an exciting idea. Thus one may summarize the findings from this purification step.

- (1) The binding subsystem consists of two components, a low molecular weight peptide and a non-protein cofactor with a molecular weight of approximately 600 or lower.

- (2) The cofactor is probably a free nitrogenous base, nucleoside or nucleotide although this is purely speculative.
- (3) In all tissues tested, the cofactor is in excess relative to the proportion of cofactor:binder necessary for the binding reaction.
- (4) Protein binder from normal tissue can be used to increase binding in malignant tissue. This suggests possible chemotherapeutic uses.

The next filter employed was the PM-30 filter. It was only employed in one experiment as it split the low molecular weight peptide between the filtrate and remainder and hence is not very useful as a purification technique. It does however suggest something about the molecular weight of the peptide. The Amicon brochure (62) states that while raffinose (mw = 594) is completely retained by this filter, bacitracin with a molecular weight of 1400 is 30% retained. As can be seen from Table VII approximately 72% of the activity was found in the filtrate. This is quite similar to the bacitracin split and would suggest that the molecular weight is similar to this compound. However, the cautions on page 86 must be re-emphasized. It should be explained as closely as possible how this experiment was performed as, since the solution was not filtered to dryness, it is important to note the actual

experimental conditions so as to ascertain whether theoretically all or almost all of the protein would be able to pass through the membrane. Approximately 20 ml of the remainder from the PM-10 filtration (i.e., the entire fraction since 40 total ml on Table IX refers to 20 ml of remainder and 20 ml of filtrate) was brought to 2 ml. (This incidentally was unintentional and was due to inattention to the apparatus.) As such, if all of the protein could pass through the membrane, 90% would have been expected to pass. The remainder was brought with water to approximately 32 mls and 12 mls were filtered. Thus one would expect approximately 4% more of the total initial protein to pass through the filter were it able to do so. This would mean that although not allowing as much protein as filtration to dryness would permit to pass through, 96% should pass if able to (theoretically). Thus, one would not expect the split as observed unless the binding protein was close to 1400 in molecular weight or else represented a structure that behaved strangely in the membrane. Hence, it may be summarized:

- (1) The PM-30 filter is a poor means of purification as it splits the total peptide between the remainder and filtrate.
- (2) The results would tend to indicate that the molecule is similar in molecular weight to bacitracin (mw = 1411).

Let us now examine filtration through the XM-50 membrane. The lowest molecular weight compound tested in the Amicon brochure that has zero retention is bacitracin (mw = 1411). The next higher molecular weight compound tested is cytochrome C with a molecular weight of 12,400. It was felt that from the previous filtrations (indicating a molecular weight around 1400) that the protein should not be retained by this filter. As such the fraction could be filtered to dryness. As is shown in Table VII there is indeed no retention of activity in the cell. However, in testing the filtrate for activity there is some loss. This can be attributed to entrapment of the molecules in the membrane. It is interesting to note that this would be a poor step to include in a good purification procedure. There is no increase in $K_p(\text{Mn})$ and in fact in some cases a decrease.

The purpose of the procedure was again to give some idea of the molecular weight of the compound. Initially it was hoped that it would be a good purification procedure, but this unfortunately does not appear to be the case.

The G-50 column as was previously mentioned proved to be a poor method of purification. However, the fact that all activity appears in bed volume indicates the molecular weight of the binder is 1500 or lower.

As can be seen from Figures 3 and 4, the Sephadex G-15 gel appears to be an excellent agent for purifying the binding peptide. It can be seen from Figure 5 that by use of the low molecular weight standards bacitracin (mw = 1411), penta-phenylalanine (mw = 825) and penta-alanine (mw = 445) that the molecular weight of the unknown peptide is approximately 1400 (extrapolated to two figures from 1350).

Conclusions--Normal versus Neoplastic Tissue

Table XIII summarizes Tables V to XII in comparing neoplastic (or carcinogen treated) tissue with its tissue of origin. The above purification steps indicate that binding is indeed due to a specific system rather than charge or low molecular weight compound effects. It can also be seen that in each step the K_p (Mn) for neoplastic tissue is lower than for its tissue of origin. The most interesting result is obtained in examining Figures 3 and 4 for the G-15 filtration. The total protein in both normal and carcinogen-treated animals is quite similar as is the percentage protein recovery (82.5 and 79.8 respectively) and percentage activity recovery (77 and 70.5 respectively) from the XM-50 filtration. However, examination of the fractions with the activity shows the normal tissue to possess approximately 1.79 times as much protein as the carcinogen-treated tissue as well as 1.65 times as much

FIGURE 5

MOLECULAR WEIGHT APPROXIMATION OF THE UNKNOWN PEPTIDE

95 The sample containing the manganese binding peptide was from animals treated with 1 mg of 3,4,9,10 dibenzpyrene in 0.1 ml peanut oil given I.P. 52 days previously.

The unknown and the standards (with the exception of bacitracin) were run simultaneously.

Each point in the figure represents the approximate value where the maximum of protein was observed.

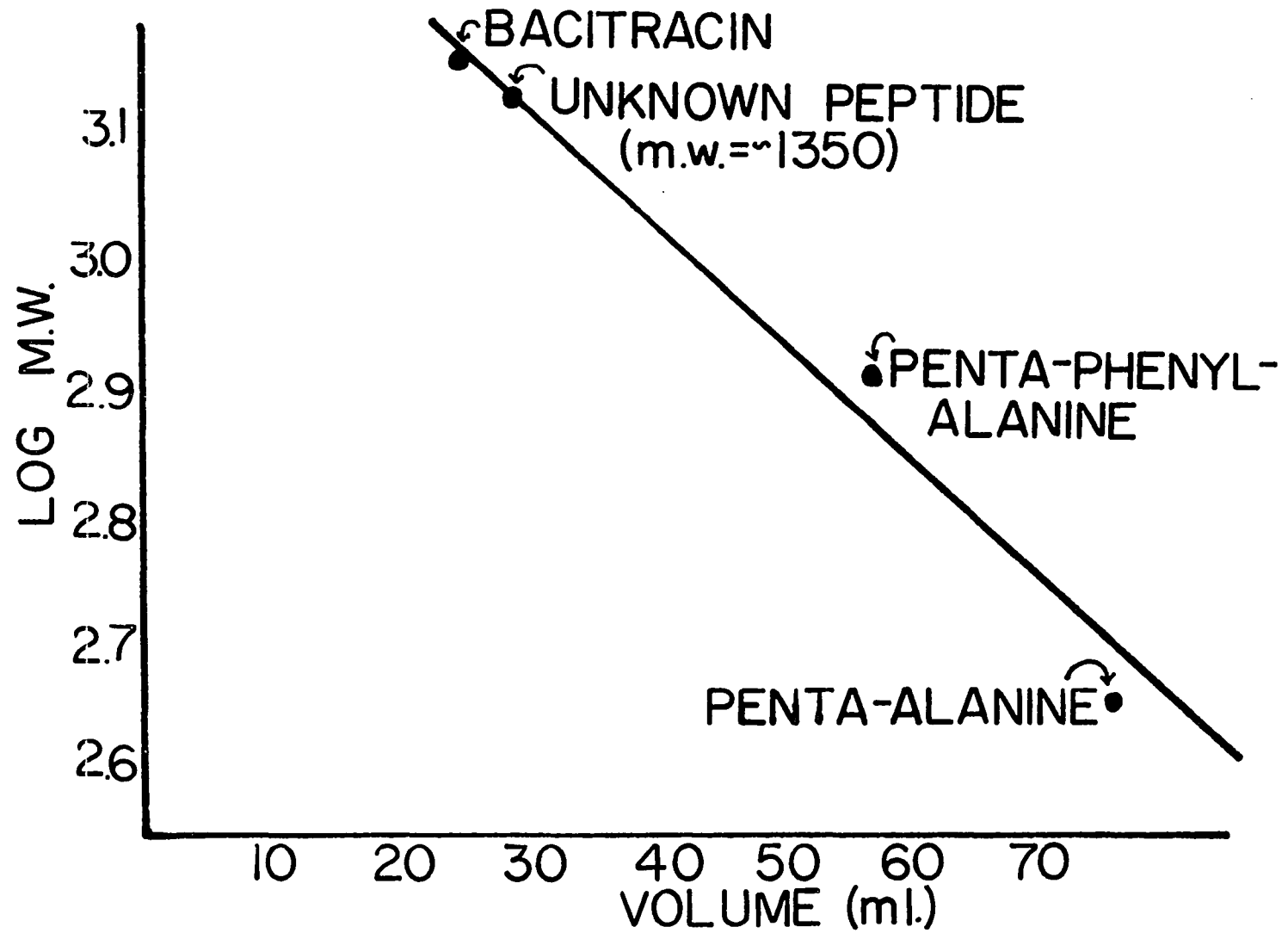


TABLE XIII

1. All values are expressed as $K_b(\text{Mn}) \times 10^{-3}$ as taken from Tables V to XII and Figures 3 and 4.
2. Other abbreviations used are the same as used in the above stated tables (see pages 63-65) except for G15-9, G15-10 and G15-11. These refer respectively to aliquots 9, 10 and 11 (3 ml each) as obtained from the Sephadex G-15 filtration.

TABLE XIII

PURIFICATION COMPARISON OF NEOPLASTIC AND NON-NEOPLASTIC TISSUE

<u>Animal Type</u>	<u>Homog.</u>	<u>Boil</u>	<u>UM-05</u>	<u>PM-10C</u>	<u>XM-50RC</u>	<u>G15-9</u>	<u>G15-10</u>	<u>G15-11</u>
Swiss--(1)	2.15	5.67	4.54	---	---	---	---	---
Swiss--(2)	2.08	5.80	5.45	4.56	---	---	---	---
Swiss--(3)	2.15	5.70	5.87	4.16	3.47	---	---	---
Swiss--(4)	2.21	5.90	5.23	4.97	3.46	7.78	25.2	13.8
8 Swiss--DBP	1.55	4.17	3.86	2.72	2.40	---	---	---
Swiss--DBP (2)	1.45	4.23	3.68	2.72	2.27	4.45	18.6	10.75
C57L/J--Liver	5.63	16.7	17.6	16.3	16.5	---	---	---
C57L/J--Hepatoma	3.28	11.58	9.26	6.40	5.18	---	---	---

activity. This would tend to indicate that the carcinogen treated tissue simply had less of the binding peptide than normal (rather than possessing an abnormal peptide with decreased binding activity). However it is too early to confirm this fact and in order to verify it, large scale preparations of the peptide from malignant and non-malignant tissue must be completely analyzed.

Purification

It is felt that an excellent purification procedure would be boiling followed by UM-05 and PM-10 filtration and Sephadex G-15 chromatography.

Indications of the Existence of a Manganese Binding Inhibitor

Before presenting and discussing the results in this section, it should be emphasized that there is no direct proof for a compound or compounds that inhibits manganese binding. Such proof could only be obtained through a complete purification as was performed with the manganese binding system. Although it is planned to perform this type of purification shortly, none has been attempted yet. The extreme lability of the inhibitor, if indeed it does exist, would make this an extremely difficult task. However, it should be pointed out that existence of the inhibitor is not in any way necessary for acceptance of the theory. However, as was pointed out (page 81), the

fact of boiled supernatant and precipitate having a greater total activity than the initial homogenate certainly indicates that an inhibitor is present. The results presented herein lend more substance to that indication.

Figure 6 shows the change in manganese binding potential in soluble fractions of various neoplastic tissues, upon heating at 50° Centigrade for various times. It can be seen that in each case there is a gradual increase in $K_p(\text{Mn})$. This phenomenon was not observed in non-neoplastic tissue.

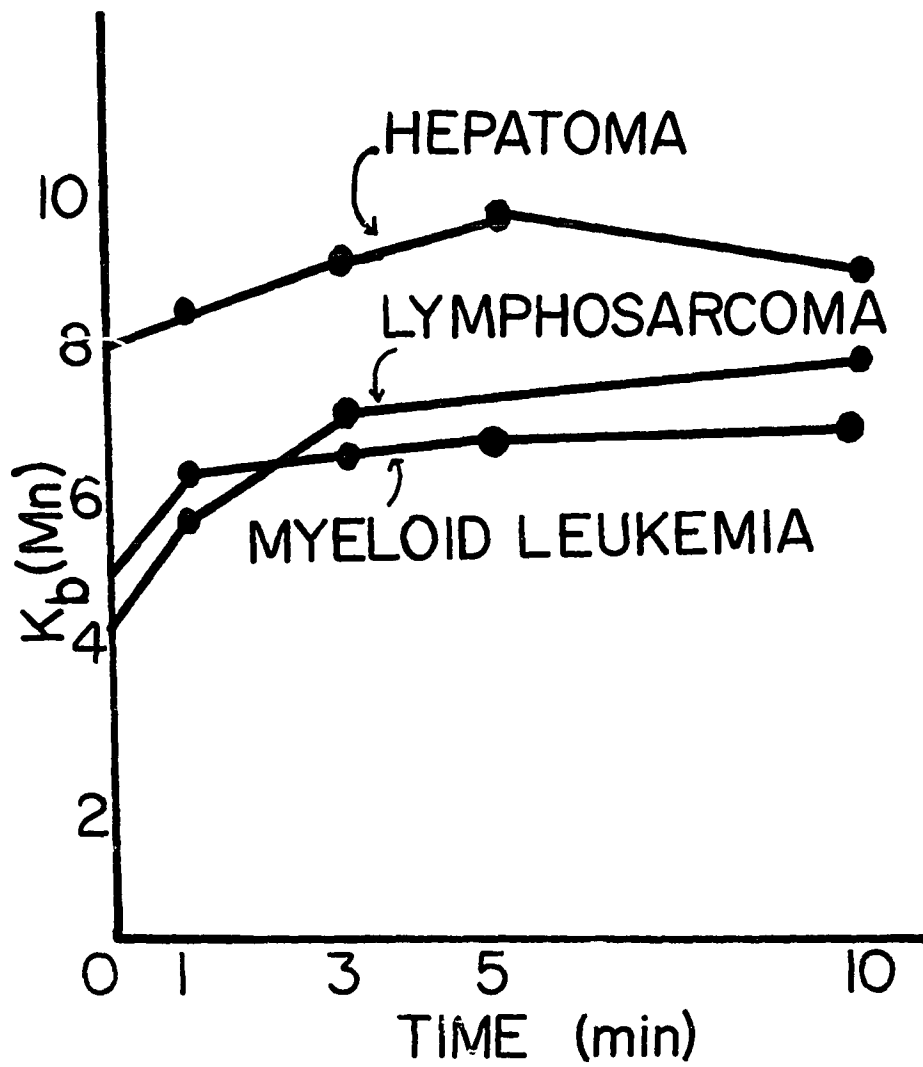
Simple calculations (Figure 6) show that the total increases do not parallel the differences between neoplastic tissue and tissue of origin as is described in Table I. It should be noted that the tissues described in Figure 6 when allowed to sit 24-36 hours at a temperature of 3-7° Centigrade and then tested, showed increases in binding potential similar to those observed after the tissues had been kept at 50°C for 10 minutes.

This would indicate the presence of an exceedingly labile inhibitor of binding which thus far has only been detected in neoplastic tissue. However it is again stressed that there is necessity of caution in interpretation of results. Much care has been taken in discussion of the binding system to determine that the effects observed were not due to artifacts. This was only

FIGURE 6

MANGANESE BINDING POTENTIAL IN NEOPLASTIC TISSUES
AT 50°C AS A FUNCTION OF TIME

The soluble fractions of the tissues listed in the figure were placed in a 50°C water bath for the times listed. The manganese binding potential (i.e., $K_b(\text{Mn})$) was measured at each time tested. Hepatoma refers to the tumor derived from the C57L/J strain. In like manner lymphosarcoma and myeloid leukemia are from the C3H/Hej and C57Bl/6J strains respectively. Further description of these systems is given on page 100.



accomplished by detailed purification of the binding system. The same must be done for the postulated inhibitor. This, as was previously mentioned, will probably be a project of momentous proportions due to the agent's extreme lability (if it actually exists). However it is not felt that the validity of the theory rests on the existence of this inhibitor, although it is certainly felt that the evidence points to the existence of such an agent or agents. Of primary importance is isolation and complete characterization of the low molecular weight protein and cofactor of the manganese binding system. Then a detailed study of the effects observed in this section is in order.

Manganese Binding Potential in Carcinogenesis

An attempt was made to stress in the introduction to this dissertation that even though the cancer cell may be derived from a given tissue of origin, the two cells must now be considered as separate moieties. Because neoplastic cell possesses a different characteristic or characteristics from its tissue of origin in no way indicates that this has anything to do with carcinogenic transformation.

The fact that manganese binding potential is lower in tumor cells than in their tissue of origin is simply an interesting fact and no more can be said unless it can be established that the change in potential occurred during

carcinogenesis. Even the isolation of the manganese binding system only shows that this hitherto undiscovered system is present. It in no way implicates it in cellular control. But if manganese binding potential is shown to drop during carcinogenesis, rather than after, one would certainly have strong evidence for considering it as a cellular control mechanism which is altered during the carcinogenic process. However, it is still necessary to point out that even with this evidence, there is no proof that it is decrease in manganese binding potential (by altering the binding system) that is the secondary cause of neoplastic transformation. As an example, a carcinogen could act on two processes in the cell, one of these being the manganese binding system. Yet it could conceivably be that it was the other process that was altered which was primarily responsible for cellular control. It is strongly felt that it is the alteration of the manganese binding system which is primarily responsible for neoplastic transformation (i.e., secondary carcinogenesis induced by a primary carcinogen, virus, etc.). However, all too often conclusions are arrived at and theories are claimed as "facts" before this has in reality been established. This has been stressed as it is felt that this point cannot be overemphasized.

In this section the results of three different carcinogenesis experiments will be presented. Statistical

analysis establishing the validity of the results will be performed and gross anatomical evidence for tumor development will be discussed. Control data showing that the effect is not a simple toxicity or traumatized reaction will also be included.

Myeloid Leukemia Implantation

It was felt that since the myeloid leukemia subcutaneously implanted had liver indicated as its tissue of origin, the implantation of a small piece of tumor directly into the liver should yield massive tumor development. This was found to be the case with widespread neoplasia observed followed by ascitic fluid formation and death in approximately 12 days. Manganese binding potential was tested daily after implantation of the tumor. Sham operated and normal mice were run on some of the same days as tumor implanted mice in order to correct for the possibility of machine errors. It has been suggested, however, that the most accurate control sham would be implantation of a piece of non-neoplastic tissue. This point must be stressed in interpretation of the results. In all cases, both the implanted and non-implanted lobes were tested. The results are shown in Figures 7-10. It should be noted that the sham operated mice have manganese binding potential in the same range as normal non-implanted C57Bl/6J mice. This clearly shows that change in the

FIGURE 7

K_b (Mn) VERSUS TIME FOR FIRST MYELOID LEUKEMIA CARCINOGENESIS
EXPERIMENT--IMPLANTED LOBE

Each point represents one animal implanted in the liver with myeloid leukemia on day zero and tested for K_b (Mn) on the day indicated.

(S) and (P) refer to the soluble and precipitate fractions from the manganese binding assay system as is described on page 31.

The hosts are C57Bl/6J female mice of approximately 3 months of age implanted with tumor which had been implanted 7 days earlier.

For a more complete description of the system, see page 46 of the text.

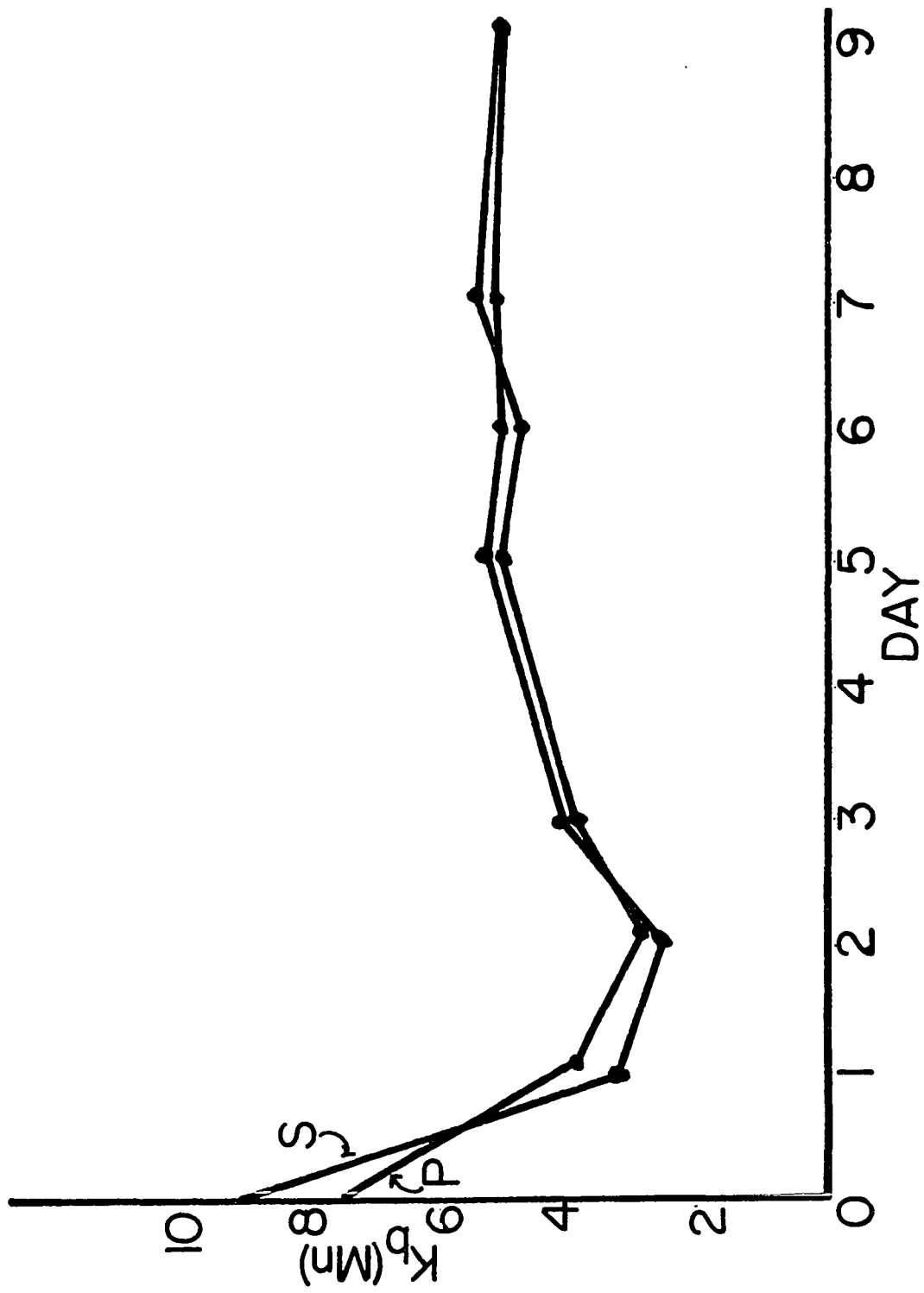


FIGURE 8

K_b (Mn) VERSUS TIME FOR FIRST MYELOID LEUKEMIA CARCINOGENESIS
EXPERIMENT--NON-IMPLANTED LOBE

Parameters utilized are the same as stated in Figure 7.

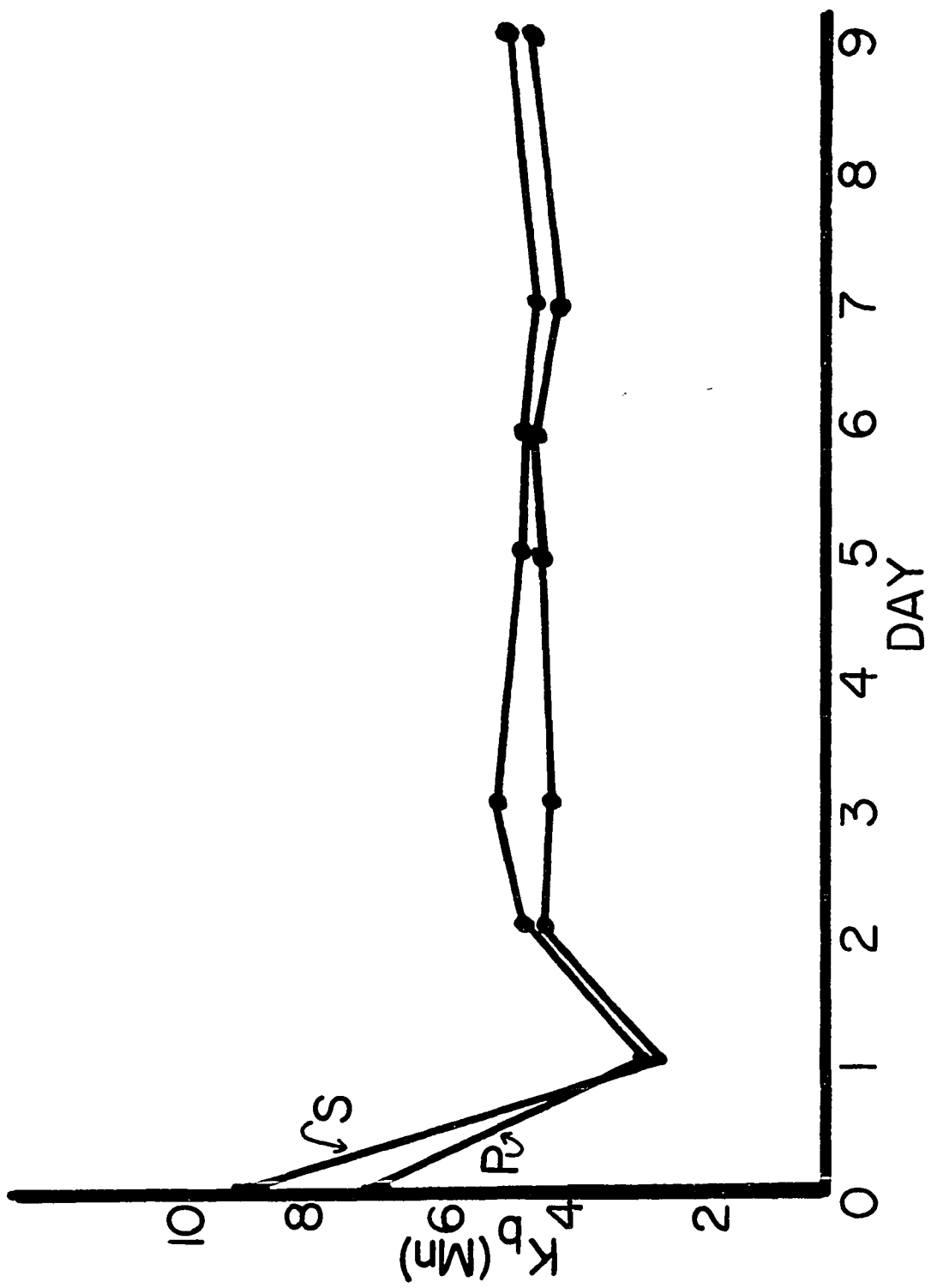


FIGURE 9

K_b (Mn) VERSUS TIME FOR SECOND MYELOID LEUKEMIA CARCINOGENESIS
EXPERIMENT--IMPLANTED LOBE

Parameters utilized are the same as stated in Figure 7.

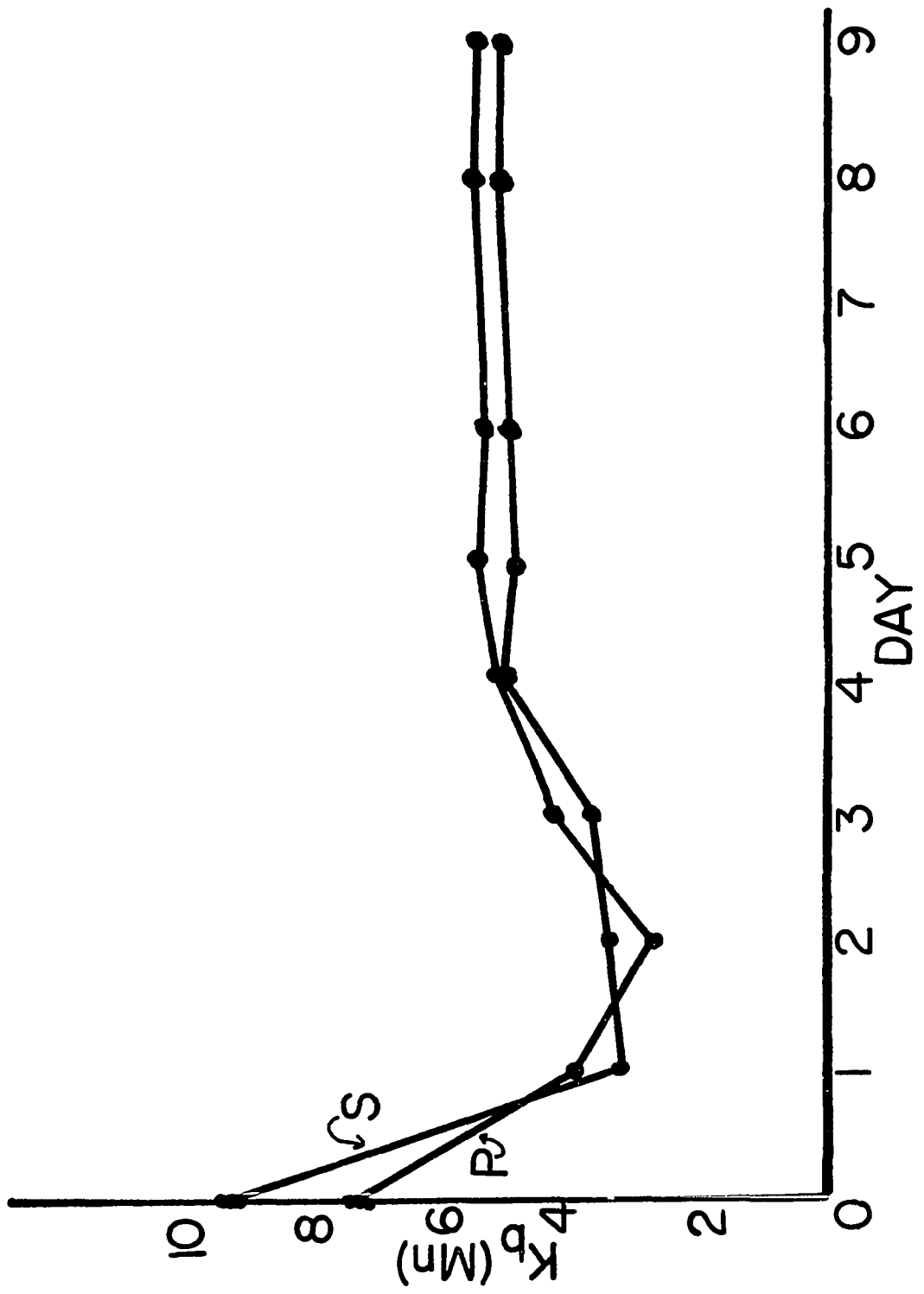
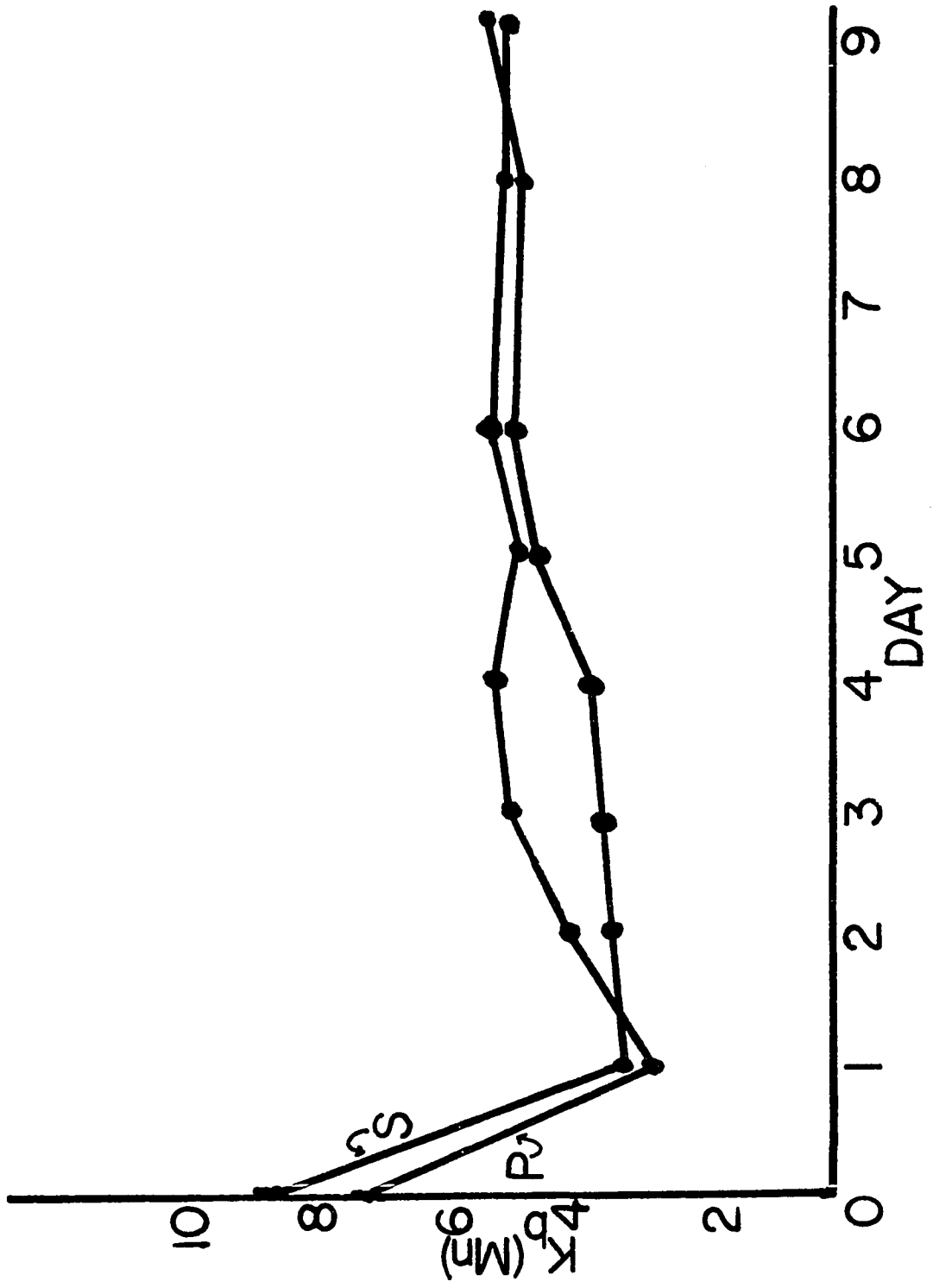


FIGURE 10

K_b (Mn) VERSUS TIME FOR SECOND MYELOID LEUKEMIA CARCINOGENESIS
EXPERIMENT--NON-IMPLANTED LOBE

Parameters utilized are the same as stated in Figure 7.



$K_b(\text{Mn})$ observed upon implantation is not due to traumatization. Examination of the figures from both experiments shows a similar pattern of $K_b(\text{Mn})$ change with time. There is a large initial drop of $K_b(\text{Mn})$ followed by a gradual rise to a plateau. However, the final $K_b(\text{Mn})$ is much lower than the $K_b(\text{Mn})$ for normal and sham operated mice. And in fact comparison of $K_b(\text{Mn})$ for myeloid leukemias and final $K_b(\text{Mn})$ achieved in these implantation experiments show these to be quite close. It is important to note that the pattern observed is similar in both implanted and non-implanted lobes. Clearly, the implantation of myeloid leukemic cells in one lobe of the liver causes similar changes in $K_b(\text{Mn})$ both in implanted and non-implanted lobes. Since the ratio of malignant to non-malignant cells in the first days of the experiment is quite low, it is obvious that the presence of malignant cells causes some change in the ability of the non-malignant liver cells potential to bind Mn. One could make many speculations as to how this is occurring, certainly one of the most intriguing being viral transmission. However, no matter what the source of change is, clearly the non-tumor cells must be affected. One might rightfully object that it is unjustified to claim that simply because the normal liver cells must in some way have their manganese binding ability altered they are undergoing carcinogenic

transformation. There is much validity to this point. The question of the validity of shams not involving implantation of a piece of non-malignant tissue must also be considered. However as will be shown in the following experiments, a similar pattern of $K_b(Mn)$ change is observed in cells treated with a chemical carcinogen where no malignant tissue was added. It is felt that in this light, the above experiments add support to the hypothesis.

Swiss Mice Given 3,4,9,10 Dibenzpyrene Intraperitoneally

The mice was given one intraperitoneal injection of 1 mg 3,4,9,10 dibenzpyrene in 0.1 ml of peanut oil. This carcinogen was used at the suggestion of Dr. Donald A. Clarke and is known to produce malignancy in a variety of organs in a short period of time. The first gross anatomical signs of malignancy were noted 42 days after injection and by 60 days all animals had these manifestations with ascitic fluid containing large amounts of malignant cells.

Lungs and livers from these animals were observed at various times after administration of the carcinogen and $K_b(Mn)$ was calculated. The results are shown in Figures 11 and 12. It can be clearly seen that a pattern quite similar to that observed with the myeloid leukemia experiment is observed. The soluble fraction from lungs does show a distinct difference. Rather than having, after the large drop on day one, a gradual rise to the plateau, there is a

FIGURE 11

K_b (Mn) VERSUS TIME FOR SWISS MICE GIVEN
1 mg 3,4,9,10 DIBENZPYRENE I.P. LIVER

Each point represents one animal given approximately 1 milligram of 3,4,9,10 Dibenzpyrene in 0.1 ml of peanut oil I.P. on day zero and tested for K_b (Mn) on the day indicated.

S and P refer to soluble and insoluble fractions as has been used throughout this dissertation.

NS and NP refer respectively to the soluble and precipitate fractions of controls given 1 mg of the liver toxin Naphthyl Isothiocyanate in 0.1 ml peanut oil.

RS and RP refer respectively to the soluble and precipitate fractions of regenerating liver controls (see text).

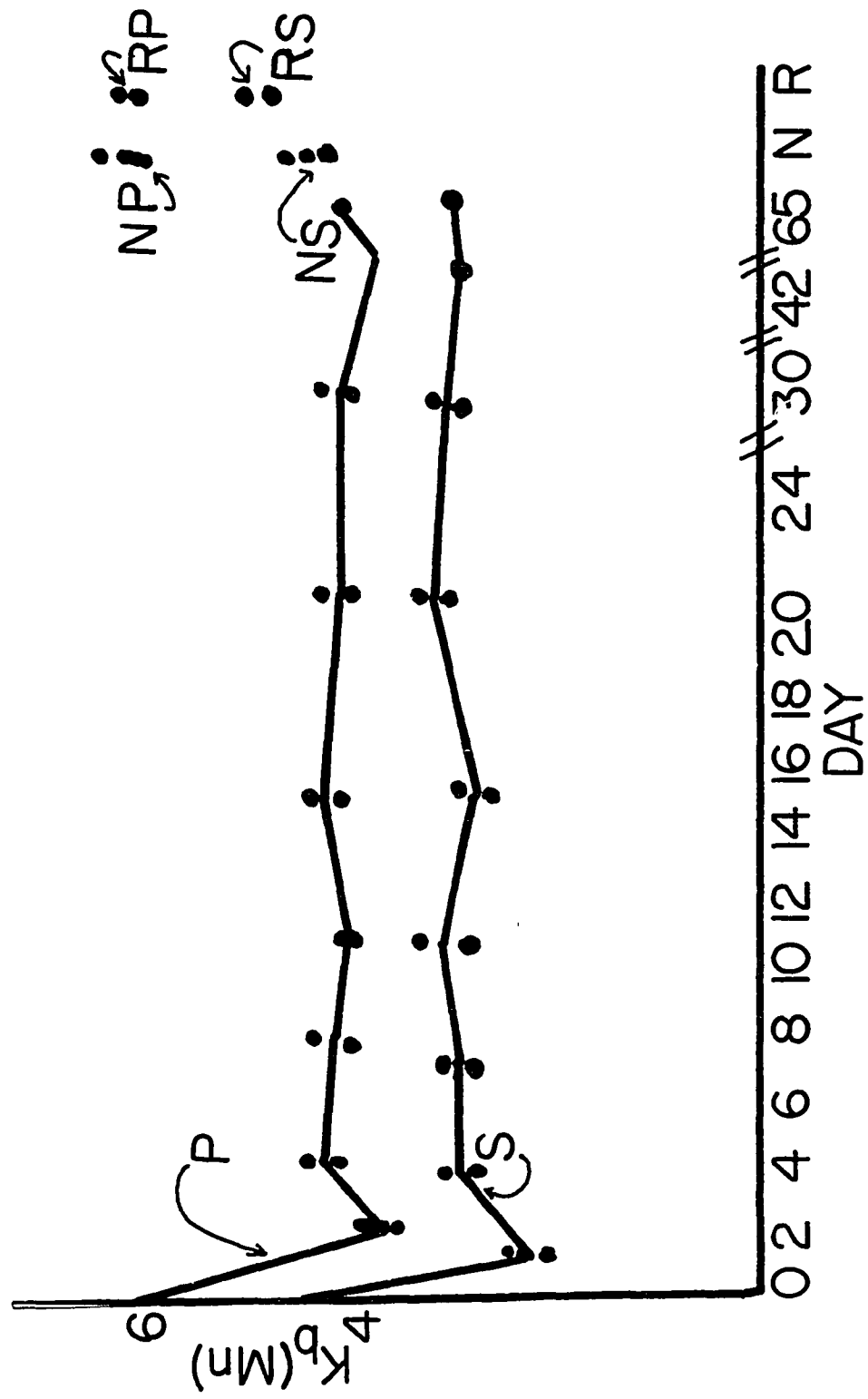
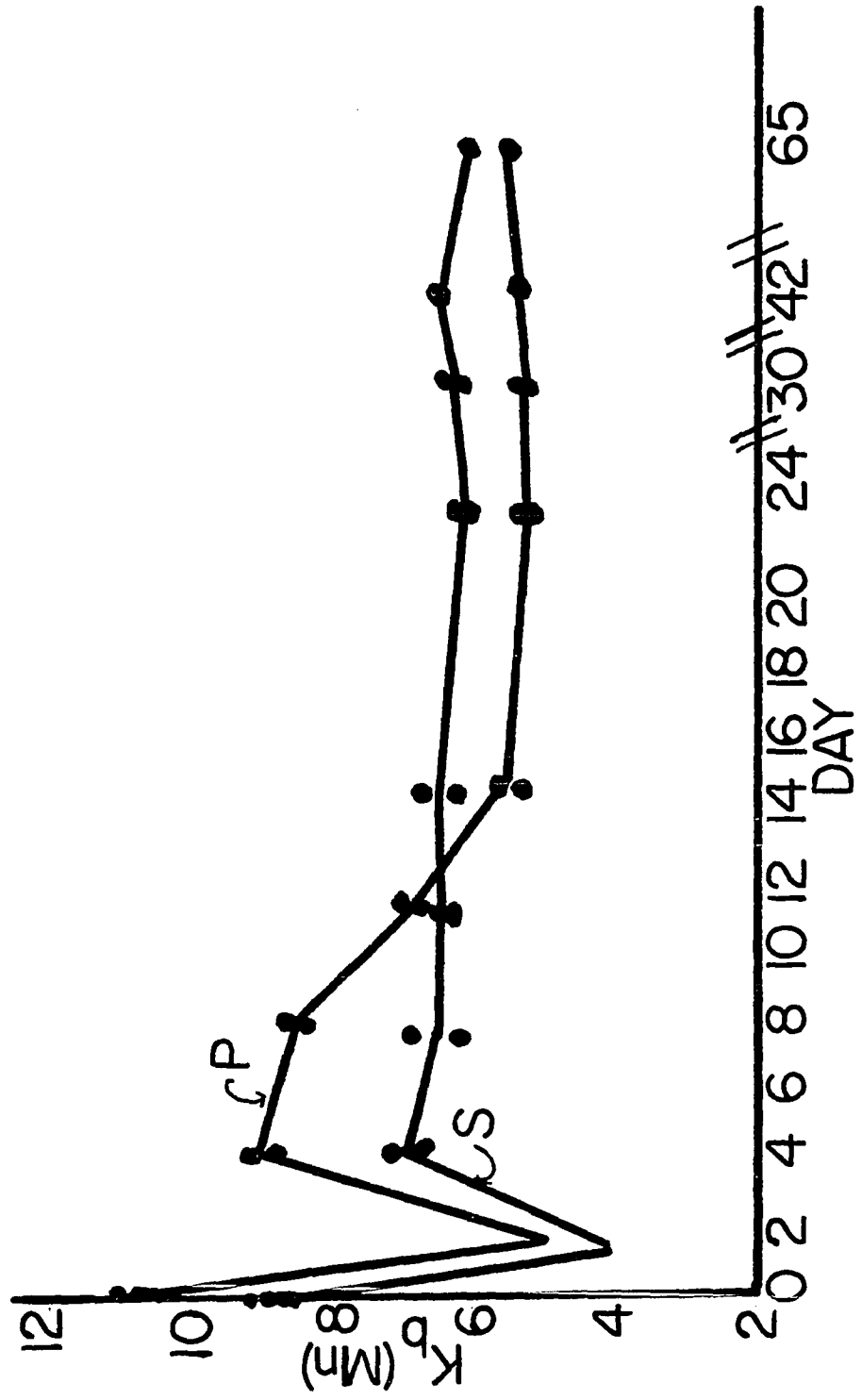


FIGURE 12

K_b (Mn) VERSUS TIME FOR SWISS MICE GIVEN
1 mg 3,4,9,10 DIBENZPYRENE I.P. LUNG

Each point represents one animal given approximately 1 milligram of 3,4,9,10 Dibenzpyrene in 0.1 ml of peanut oil I.P. on day zero and tested on the day indicated.

S and P refer to soluble and insoluble fractions as has been used throughout this dissertation.



rise to normal and then sharp drop to the plateau. This represents the one major deviation that has been observed. It should be noted that one animal tested on day 4 but not included in the figures showed perfectly normal readings for lung and liver. It is assumed that this animal either received a very low dose of carcinogen or none at all. It must be pointed out that when injecting a suspension like this, many times the hypodermic needle becomes filled with solid and the animal receives little or none of the compound. A new needle must be employed in this case. Attempts were made to discard any mice in this category, but it is conceivable that one was inadvertently overlooked. One might postulate that this one mouse might be one of the few resistant to tumor development. While this is a distinct possibility, one would tend to reject it on the grounds that all animals remaining after 60 days showed neoplasia. However, the important point, it is felt, is the establishment of the pattern of $K_p(\text{Mn})$ versus time similar to that observed with the myeloid leukemia carcinogenesis. However, one might question whether even two different means of carcinogenesis yielding similar patterns is significant. To check this, a third means of neoplastic induction as is described below was attempted.

Before delving into this, one should point out one more vitally important point contained in Figures 11 and 12.

It can be seen that animals treated with Naphthyl Isothiocyanate showed K_p (Mn) values like that of non-carcinogen treated mice. This compound is an extremely toxic liver poison. Whereas if the effects observed with 3,4,9,10 dibenzpyrene were simply toxicity effects one might not expect any changes of K_p (Mn) in the lungs of animals treated with Naphthyl Isothiocyanate, one would surely expect changes in the liver. The fact that no changes were observed clearly indicates that the pattern observed is not simply due to toxicity of the carcinogen.

One final control must be mentioned. One can conceive of many instances where there is rapid proliferation of cells but no neoplasia. Notable examples would be fetal development and healing of wounds. It is most important to determine whether the change in K_p (Mn) pattern observed is simply a function of rapidly proliferating cells or primarily a function of neoplastic development.

One rather simply induced case of rapid proliferation without neoplasia is that of regenerating livers. By excising approximately two-thirds of the liver, it can be shown that the remaining liver begins rapid mitotic division with a peak of activity reached in 24-48 hours after the operation. It can be seen from Figure 13 that regenerating livers (taken and tested for K_p (Mn) 30 and 72 hours after excision of liver) showed no appreciable

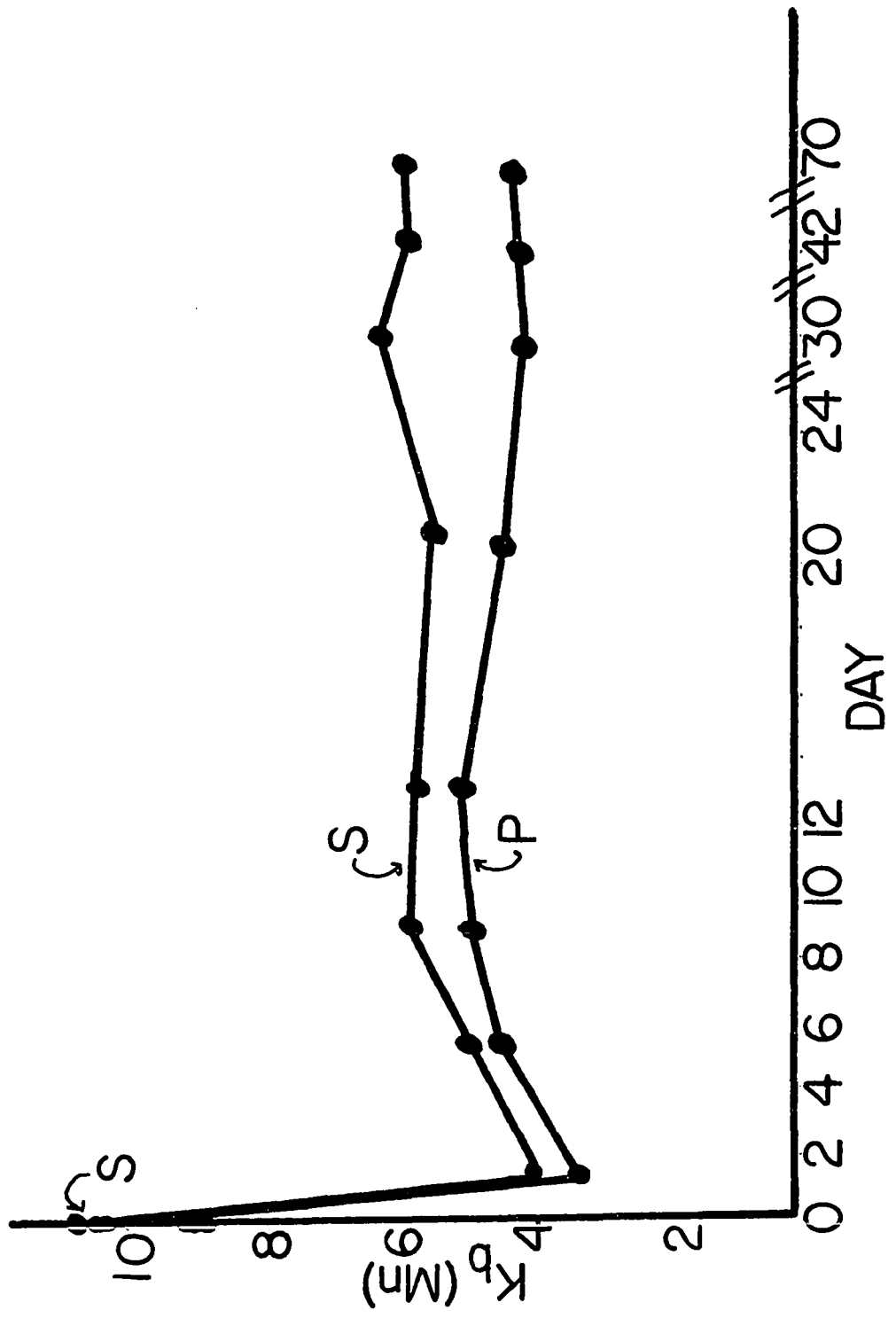
FIGURE 13

K_b (Mn) VERSUS TIME FOR A/Hej MICE GIVEN
0.24 mg 3,4,9,10 DIBENZPYRENE I.V.

Lung

Each point represents one animal given approximately 0.24 milligrams of 3,4,9,10 Dibenzyrene in acetone-water carrier I.V. on day zero and tested on the day indicated (see page 30 of the text).

S and P refer to soluble and insoluble fractions as has been used throughout the dissertation.



difference from $K_p(\text{Mn})$ of normal Swiss mice. The 30-hour mouse tested was in the most active state of mitotic division, and in the 72-hour mouse, while not at a peak, rapid mitotic division was occurring. This is certainly strong evidence that the pattern observed of change in $K_p(\text{Mn})$ as a function of time is inherently related to the carcinogenic process rather than simply being a characteristic of rapid mitotic division.

A/Hej Mice Given 3,4,9,10 Dibenzpyrene Intravenously

This strain of mice is known to be very susceptible to pulmonary tumors. Andervont (6) found that this strain of mouse given approximately 0.25 of the carcinogen 1,2,9,10 dimethylbenzanthrene I.V. develops pulmonary tumors in 100% of the cases in 8 weeks. Using a variation of Andervont's technique as described (page 30), A/Hej mice were given approximately 0.24 mg of 3,4,9,10 dibenzpyrene intravenously. $K_p(\text{Mn})$ from excised lung tissue was measured at various intervals. The results are shown in Figure 13. As can be seen the pattern of $K_p(\text{Mn})$ change is quite similar to those previously observed. Visual observation of mice injected 70 days previously showed nodules resembling pulmonary tumors. This experiment represents the third tumor induction method where the pattern previously described was observed.

Statistical Analysis of Results

As in these carcinogen experiments a large number of mice were not employed; it is essential to perform statistical analysis on the results in order to determine if they are significant. Since the number of samples is so small one cannot apply a normal distribution equation but rather must rely on Student's t distribution. Briefly this method involves calculating the value t by the formula

$$t = (x - \bar{x})/s$$

where x is the normal mean, \bar{x} is the number one wishes to determine the significance of and s is the standard deviation. One then employs a table relating t to various degrees of freedom where degrees of freedom is defined as the total number of samples in the normal populace tested minus 1. One can then determine a probability value, P. This probability value can be expressed as the number of chances per 100 or 1000 of having the \bar{x} in question being a simple variation from the normal populace and not of significance. It is generally accepted that if the probability is less than or equal to 5 chances per 100 that the \bar{x} is a significant number.

In the case of the carcinogenesis experiments previously described, x refers to the mean K_p (Mn) of normal non-carcinogen or implanted mice tested. \bar{x} (with the

exception stated below) refers to the highest number of $K_p(\text{Mn})$ shown after the value reached a plateau. As an example it can be seen by examining figure 12 that the highest value for $K_p(\text{Mn})$ after a plateau (approximate) has been reached is approximately 6.1 (actual value is 6.11). This occurs at day 30. Hence if this number is shown to be significant, all others lower than this (and hence further from the normal) will also be significant.

The one exception to this involved the calculations of Swiss mouse lungs (soluble fraction) of 3,4,9,10 dibenzpyrene treated animals. This was the only case where one saw the initial drop, a sharp rise and then another drop to the plateau. The important question was whether the rise was significant. Hence the value of $K_p(\text{Mn})$ occurring at the apogee (day 4) of the rise was employed in calculation of t . This number proved to be significant. It is thus obvious that all numbers lower than this are also significant.

Table XIV shows the results of these statistical calculations. It can be seen that in all cases, the probability value is less than 50 parts/1000. Thus it can be concluded that the results as presented do have statistical significance with regard to decrease of $K_p(\text{Mn})$ in carcinogenesis.

TABLE XIV

1. The values x , \bar{x} , s , t and Prob. are as described in the text (page 126).
2. No. refers to total number of normal animals tested.
3. (S) and (P) are soluble and precipitate fraction as has been referred to throughout the text.
4. The first set of values for myeloid leukemia refers to Figures 6 and 7 while the second refers to Figures 8 and 9.
5. DBP refers to 3,4,9,10 dibenzpyrene. The text should be consulted for details of administration.

TABLE XIV

STATISTICAL ANALYSIS OF CARCINOGENESIS EXPERIMENTS

Experiment and Tissue Tested	No.	x	\bar{x}	s	t	Prob.
Myeloid Leukemia--Implant Lobe (S)	4	9.03	5.32	0.234	15.82	0.01
Implant Lobe (P)	4	7.35	5.15	0.450	4.89	0.1-0.2
Non-Implant Lobe (S)	4	9.03	5.33	0.234	15.78	0.01
Non-Implant Lobe (P)	4	7.35	5.22	0.450	4.73	0.1-0.2
Myeloid Leukemia--Implant Lobe (S)	4	9.06	5.25	0.400	9.53	0.01-0.1
Implant Lobe (P)	4	7.15	5.25	0.430	4.42	0.2
Non-Implant Lobe (S)	4	9.06	4.64	0.400	11.25	0.01
Non-Implant Lobe (P)	4	7.15	5.10	0.430	4.77	0.1-0.2
Swiss-DBP Treated Liver (S)	5	4.20	3.33	0.245	3.55	0.2-0.5
Liver (P)	5	5.87	4.26	0.240	5.88	0.1
Lung (S)	5	10.98	9.90	0.36	5.77	0.1
Lung (P)	5	8.80	6.70	0.387	5.42	0.1-0.2
A/Hej-DBP Treated--Lung (S)	5	10.53	6.11	0.427	10.34	0.01-0.1
Lung (P)	5	9.45	6.35	0.283	10.96	0.01-0.1

Manganese Binding Potential of Various Proteins and Enzymes

Although it is known that there are enzymes which bind manganese and their substrates in a ternary complex and hence should give a decrease in the ESR signal of the ion, it is of academic interest to note whether a decrease of average peak height is observed when the ion is added to a concentrated solution of the enzyme or other proteins. Although this is in no way an attempt to compare these compounds per se with the manganese binding system (as the system is composed of a peptide and cofactor), if there were significant binding, it would tend to decrease the importance of the binding system.

Hemoglobin (5 mg/ml), bovine plasma albumin (5 mg/ml), creatine phosphokinase (2 mg/ml) and isocitric dehydrogenase (10 mg/ml-dialyzed) were tested for their effect on decreasing the manganese signal of the 0.006 molar standard that has been used for the majority of studies cited in this dissertation. In no case was any decrease in signal observed. Certainly had lower concentrations of manganese been used, a decrease might have been observed. But this study was not necessary as even were a decrease observed it would only verify what the above showed; these proteins either have much less potential to bind the ion than does the manganese binding system or when the ion is bound there is no or little loss of signal.

A criticism that might be leveled is that normally a protein such as isocitric dehydrogenase has TPN as a cofactor and a more suitable control would be to have the cofactor present. Simply because of the high molecular weight (i.e., 64,000) of this enzyme one might suggest that it would have less potential to bind than does the binding system proposed in this dissertation.

As one last comment on this matter, it was shown that addition of the cofactor component (PM-10 filtrate) to the above enzyme and non-enzyme protein solutions caused no appreciable binding. It would thus appear that the cofactor is not a non-specific agent causing binding of any available protein (or any protein requiring manganese), but rather might possibly be looked upon as a somewhat specific agent for the peptide discussed in the purification procedure.

It is conceded that the results in this section simply suggest certain things. However, while once again offering no proof for the theory, the results are in line with same and do nothing to contradict it. It is hoped in the near future to perform complete studies as to the relationship of the binding system to enzymes such as isocitric dehydrogenase.

Final Conclusion

Since throughout this dissertation it has been attempted to analyze each point in detail, this conclusion will be brief so as to avoid repetition.

It is felt that the evidence in this dissertation strongly indicates manganese binding to be an important cellular control mechanism that is broken down in neoplasia. The word indicates cannot be over-emphasized. To establish the theory proposed, as fact, will require a great deal of work. It is hoped that the theory can be used to find useful anti-cancer chemotherapeutic agents. Preliminary studies suggest this possibility. But this too will require much effort. However, it is felt that the evidence presented herein is sufficient to warrant more investigation of manganese binding in cellular control either to prove or disprove its importance.

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