EXPERIMENTAL STUDIES ON BONE MARROW HYPOPLASIA ASSOCIATED WITH DRUG ADMINISTRATION

by

Sharad Kumar, M.B., B.S.

A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of Master of Science.

Department of Medicine

University of Utah

June, 1954

This Thesis for the M.S. degree

by

Sharad Kumar, M.B., B.S.

has been approved by

Chairman, Thesis Committee.

Head, Major Department.

Dean, Graduate School.

ACKNOWLEDGEMENTS

I wish to express my utmost gratitude to Dr. G. E. Cartwright for his guidance, constant help and criticism in the preparation of this thesis and the experimental work leading to it. He has never failed to stop his work to answer a question for me.

To Dr. M. M. Wintrobe, I am indebted in many ways: for his personal interest in me and my work, his encouragement, teaching, and above all the inspiration which drew me all the way from Lucknow to Salt Lake City.

To Dr. A. J. Samuels, I would express my sincere thanks for his advice and help on innumerable occasions. Gratitude is also extended to those of the departmental staff who assisted in many ways, particularly to Helen Ukradyha, Betty Krumm and Patricia Gee whose cooperation made this task particularly pleasant.

TABLE OF CONTENTS

I. INTRODUCTION

A. Review of Literature.

Recognition of the fact that certain drugs may produce temporary or even permanent alterations in the hemopoietic tissues following the intermittent administration of a therapeutic dose is relatively recent. In 1922, Shultz (1) described five fatal cases with severe sore throat, marked prostration and an extreme reduction in the number of circulating granulocytes -- a syndrome now recognized as agranulocytosis. Plum (2) next drew attention to the relationship between the rising incidence of agranulocytosis and the increasing availability of amidopyrine. Subsequently, the causal relationship between amidopyrine and agranulocytosis was demonstrated by Madison and Squier (3), who showed that the administration of a single dose of amidopyrine to each of two patients who had recovered from agranulocytosis, was followed by a rapid fall in granulocytes.

Since then, a number of drugs have been incriminated as the cause of certain abnormal alterations in the blood following the intermittent administration of therapeutic doses. These changes may involve erythrocytes, leukocytes and platelets in the blood, or their precursors in the bone marrow. (Table I) A striking feature is the fact that the disease develops only in an extremely small proportion of the cases exposed to the drug.

As an example of the involvement of erythrocytes, certain hemolytic anemias are known to be allergic in origin. The anemia associated with Favism, a disease caused by the broad bean Vicia faba, is hemolytic in nature. Following previous sensitization, there are evidences of a rapid, extensive intravascular hemolysis one or two hours after a recent exposure

Table I*

COMMON DRUGS ASSOCIATED WITH BLOOD DYSCRASIAS

Group		Pancytopenia	Agranulocytosis	Thrombocytopenia	Anemia Hemolytic	Anemia Aplastic
Analgesics	Butazolidine	X	X	X	X	
	Sodium Salicylate				X	
	Amidopyrine		X			
Anti-Convulsants	Mesantoin	X				X
	Paradione	X				
	Phenurone	X				X
	Thiantoin	X	X			
	Tridione	X	X			X
Anti-Histaminics	Benadryl				X	
	Diatrin		X			
	Pyribenzamine		X			
Anti-Infectives	Atabrine					X
	Chloramphenicol	X	X			X
	Para-aminobenzoic acid			X		
	Penicillin		X			
	Plasmochin		X		X	
	Primaquine		X		X	
	Promin				X	
	Sulphonamides	X	X	X	X	X
	Gantrisin		X	X		
	Streptomycin			X		X
Anti-Thyroids	Methyl thiouracil		X			
	Propyl thiouracil		X			
	Tapazole		X			
	Thiouracil		X			
Hypnotics	Allurate		X	X		
	Amytal	X				
	Phenobarbital		X	X		
	Presidon		X			
	Sedormid			X		
Miscellaneous	Apresoline	X				
	Myanesin				X	
	Pronestyl		X			
	Stilbestrol			X		
	Quinidine			X		

*Modified after Fitz-Hugh Jr. (15)

to the antigen. The manifestations include fever, malaise, vomiting, pain in the back and rapidly developing anemia. Bhagdad spring anemia is a similar disease precipitated by exposure to the pollen of spring flowers after initial sensitization.

Drugs which are known to cause only leukopenia and granulocytic hypoplasia of the bone marrow are of many types and chemical structure (Table I). Thrombocytopenia has also been reported to follow the administration of certain drugs (Table I) including "sedormid" (4, 5, 6, 7) and quinidine (8). Many drugs have been claimed to cause hypoplasia or aplasia of the bone marrow. Among these, reported more recently are, "Mesantoin" (9) and chloramphenicol (10). The first report of any significant influence of chloramphenicol upon the hemopoietic system was made in 1949 (11). Since that time approximately 100 cases of blood dyscrasias have been associated with the administration of this drug (10, 12). The subject of drug-induced hemopoietic disorders has been reviewed recently by a number of workers (13, 14, 15, 16).

B. Current Concepts of the Mechanism of Drug-Induced Hematologic Reactions.

1. Significance of the benzene ring:

Prior to 1930, the main concept evoked to explain the hematologic alterations following administration of certain drugs was that of a direct toxic effect. Benzene typifies the drug that has been extensively investigated in this respect. In man a number of cases of aplastic anemia following benzol intoxication have been reported (17, 18, 19, 20). Analysis of the histories of the patients exposed to various benzene-containing industrial products reveals a great individual variation in their response. Not infrequently, patients exposed to a relatively small dose have died,

while others exposed over a longer period of time and to higher concentrations of the drug, have survived and indeed, have had no symptoms. The same phenomenon has been observed in experimental animals that have received benzene. The immediate disappearance of leukocytes from the circulation and their absence in the capillaries throughout the body in experimental animals, together with the appearance of degenerated forms of granulocytes in the blood, led Selling (21) to conlude that the action of this agent on the blood cells was of a toxic nature. He considered individual susceptibility to be a strongly determinant factor. Studies on the regeneration of bone marrow in benzene intoxicated animals showed that after hypoplasia or aplastic alterations, the bone marrow recovered only to the point of primitive reticular cells (22).

Kracke (23) in 1932, drew attention to the presence of the "benzamine" ring in many compounds alleged to cause blood dyscrasias. He suggested that the presence of this structure might be responsible for causing the hematologic abnormality. He supported this hypothesis by demonstrating once again that aplastic anemia and death may follow the administration of benzene in experimental animals, confirming the work of Brune (24), Selling (25) and Fontana (26). Kracke concluded that granulocytopenia in particular was due to the effects of one or more of the oxidation products of benzene. Among the products suggested were cathecol and hydroquinone. However, he did not succeed in demonstrating that such an effect followed the administration of these compounds.

Kracke's hypothesis does not seem valid for several reasons. It has been estimated from the number of cases reported that of all the patients given amidopyrine only 0.86 per cent develop a sensitivity reaction involving

hemopoietic tissues. Attempts to produce agranulocytosis by injecting or feeding amidopyrine to a number of animal species were successful only in two rabbits (3, 27). Moreover, there are a number of compounds like para-aminobenzoic acid or acetyl salicylic acid which have a "benzamine" or aminobenzene ring, and which have been in use for a number of years without being incriminated to any great extent with hematologic alterations. So far, therefore, we must conclude that there is no evidence to indicate that the 'Benzamine' ring per se is toxic to the bone marrow.

2. Metabolic Antagonism: (Inhibition)

The chance discovery (28) that β-acetylpyridine would cause signs of nicotinic acid deficiency in dogs introduced the studies of metabolic antagonism. Wooley (29) has defined this phenomenon as a mechanism by which an enzyme, vitamin or some other equally important substance may be "blocked" in its metabolic pathway. The presence of a structure which closely resembles a metabolite in its stereo-chemical characteristics may entirely abolish the function of this metabolite, if the anti-metabolite is present in sufficient concentration. Under these circumstances a "deficiency" of the metabolite may become apparent.

As an example of metabolic antagonism, folic acid may be inhibited by its anti-metabolite 4-amino-pteroylglutamic acid "Aminopterin" (Fig. 1). Such inhibition is accompanied, in experimental animals, by pancytopenia (30). Likewise, sulphonamides are known to inhibit para-aminobenzoic acid in bacterial systems.

The possibility exists that chloramphenicol may act as an anti-metabolite in producing abnormal blood reactions. Wooley (29) has shown that chloramphenicol may inhibit the growth of E. coli and that the inhibiting effect

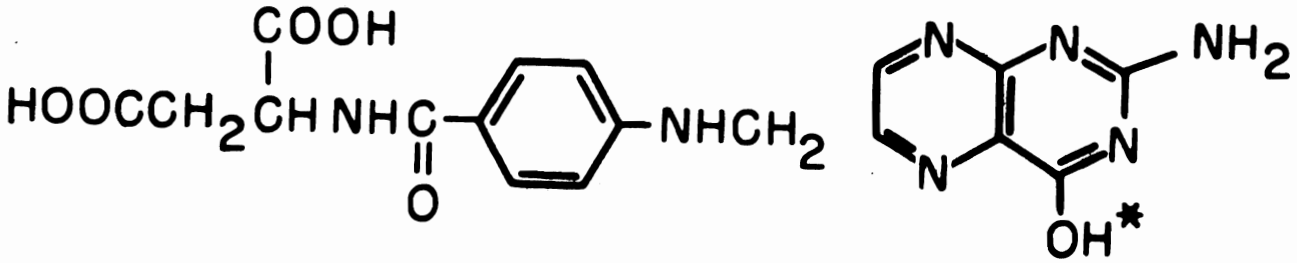


Figure 1. Structural formula of folic acid (Pteroylglutamic acid). Aminopterin (*p*-amino-pteroyl glutamic acid) is formed by the replacement of OH* with a NH_2 group.

could be overcome by phenylalanine, tyrosine, and tryptophane. More recently Bergmann (31) has shown that chloramphenicol 'blocks' the conversion of anthranilic acid to trytophane in bacterial systems employing E. coli. Thus, there is evidence that this antibiotic acts as an anti-metabolite to certain essential growth factors, at least in bacterial systems. If such a mechanism operates in human beings, it still remains to be explained why this action takes place only in approximately one out of a hundred thousand individuals to whom chloramphenicol is administered.

3. Immunologic Mechanisms:

The development of allergy requires an initial sensitizing contact with an antigen followed by re-exposure to the same antigen following a latent period. In allergy to drugs this mechanism has no resemblance to a pharmacologic or toxic effect of the drugs, since it may be, and frequently is, brought about by a relatively small dose. The allergic reaction is further differentiated by the fact that the administration of the responsible agent, even in larger doses, will provide the abnormal reactions only in a relatively few individuals.

That abnormal hematologic reactions associated with the administration of certain drugs follow an immunologic pattern is a frequent clinical observation. However, in most instances of drug-related blood disorders circulating antibodies to the drugs have not been demonstrated. There are only a few noteworthy exceptions.

In 1936, Dameshek et al. (32) demonstrated passive transfer type antibodies in patients with agranulocytosis following administration of amidopyrine. In a donor who had received an intracutaneous injection of the serum from a sensitized individual, a positive skin test was obtained using for an antigen, a mixture of amidopyrine and blood serum which had been permitted to stand

for 10 days. Moeschlin and Wagner (33) demonstrated the presence of a leukocyte agglutinating factor in the blood of individuals sensitive to amidopyrine. This factor was effective *in vitro*, and a transfusion of 300 ml. of blood from a drug-sensitive patient, taken one hour after the administration of the drug, profoundly reduced the granulocyte count in a normal subject.

An immunologic mechanism has also been demonstrated in thrombocytopenia related to drugs. By the use of appropriate studies, Ackroyd (5) demonstrated that "Sedormid" "links" with platelets to form an antigen. In each person taking "Sedormid", antibodies to this complex are allegedly formed; only in rare instances, however, is the titer of antibodies sufficiently high to cause hematologic alterations. With an adequately high titer, the antibody in the presence of complement, combines with the drug-platelet complex (antigen). This is followed by agglutination of the platelets. Thrombocytopenic purpura is thought to be the consequence of the *in vivo* destruction of the platelets. Bigelow *et al.* (34) have demonstrated the existence of a similar agglutination mechanism for platelets in patients with quinidine-induced purpura.

Experimentally, Landsteiner (35) and his co-workers showed that simple chemical compounds could be used as antigens by artificially coupling them to proteins. The specificity of such an artifical antigen is determined, in part, by the configuration of the chemical substance or haptene. Landsteiner and Jacobs (36) further showed that substances which produce skin sensitivity combine with proteins, whereas those which fail to cause skin sensitivity would not combine with the protein. This work suggested that skin sensitivity to drugs depends on their capacity to combine with a protein in the patient's body. Phillips (37) has shown that sensitization to

drugs which are injected or taken by mouth is less common than sensitization to drugs that are applied to skin. Great variations exist in an individual's susceptibility to sensitization by drugs.

Attempts to sensitize animals to drugs were made by Gerber and Gross (38) who claimed to have produced anaphylactic shock in guinea pigs by the use of sulfonamide azo-protein. The specificity of the drug was clearly suggested in these studies. Samson and Götz (39) claimed to have produced anaphylaxis in guinea pigs by the use of an incubate of amidopyrine and autologous guinea pig blood. Gell (40) in 1946 demonstrated that precipitins may be produced in the blood of rabbits following the intravenous injection of simple chemical compounds.

In summary, it appears to be established that certain drugs combined with serum or tissue protein become antigenic. The subsequent development of sensitivity, together with the readministration of the drug, in rare instances is associated with an alteration in the formed elements of blood or blood forming organs. Moreover, as was proposed in a recent editorial (41), such a sensitivity reaction can be elicited by another compound which closely resembles the chemical structure of the original sensitizing drug. Such a cross reaction has been described by Landsteiner (35) in artificially conjugated antigens. This type of reaction may indeed explain the occurrence of a drug "hypersensitivity-like" phenomenon in patients from whom no history of previous drug ingestion can be obtained.

C. Approach to Present Work.

The object of this study has been to produce changes in the blood and the bone marrow of experimental animals similar to the changes seen in human subjects following administration of certain drugs. In this effort

a variety of species and methods have been employed.

Of the large number of drugs known to cause alterations in the hemopoietic tissues, we have mainly utilized chloramphenicol. The role of chloramphenicol in causing hematologic alterations has been a subject of many recent reports (42, 43). Because the chemical structure of chloramphenicol includes the nitrobenzene group, Smadel (44) called attention to its possible toxic effect on the blood forming organs. We have attempted to "link" chloramphenicol to protein present in homologous blood or bone marrow. In spite of the fact that heterologous protein is more antigenic, we decided to use homologous protein in order to reproduce, if possible, the immunologic mechanism alleged to be responsible for these abnormal hematologic reactions in man. Also, since we were concerned with mechanisms that involve the hemopoietic tissue, we decided to combine the drug with homologous bone marrow or blood, rather than with other organs or media, in most instances. We have tried to produce anaphylaxis in guinea pigs according to the technic of Samson and Götz (39). The purpose of this study was to learn if a drug could be bound to blood in vitro and used as a sensitizing agent by so simple a procedure. If this were true for chloramphenicol, it would have been of interest to apply the method as a simple screening procedure to test the immunologic characteristics of other drugs as well.

Certain evidence appeared to support the hypothesis that blood or bone marrow may be altered by the use of homologous tissue extracts. Morgan (45) produced lesions in the central nervous system of monkeys after repeated injections of monkey spinal cord tissue. Freund (46) produced identical lesions after a single injection of homologous brain extract in guinea pigs. Cavelti and Cavelti (47) produced renal lesions following the use of homologous

kidney extracts and streptococci. For these reasons experimental animals received numerous injections of homologous bone marrow over prolonged periods of time, simulating the injection schedules employed by these investigators.

The possibility exists that it is not the drug but an unusually occurring metabolite, that combines with the tissue protein. To test such a hypothesis we have used both the "diamine" (D (-) threo-1-p-aminophenyl-2-amino-1, 3 propanediol) and palmitic ester of chloramphenicol. In addition to blood and bone marrow, combination of liver, spleen, and kidney homogenates incubated with the drug were also employed.

It has been stated (48) that the Hartley strain of guinea pigs is particularly susceptible to the development of allergic encephalomyelitis following the use of homologous brain extracts. The profound efficacy of adjuvants containing killed mycobacteria together with the intracutaneous route of injection for iso-sensitization has also been reported (48). In certain experiments we have made use of all of these observations.

Since Bjorklund and Hellstrom (49) claimed to have induced aplastic changes in the bone marrow of rabbits after injections of bone marrow antiserum produced in horse, studies on these lines have been reported (50, 51, 52, 53) with the use of anti-erythrocytic and anti-leukocytic sera. We also studied the effect of anti-bone marrow serum in different species of animals in order to explore further possible means of inducing bone marrow hypoplasia.

Studies were also conducted in which chloramphenicol and its congeners were fed to young rats in varying concentrations in the diet. To study the influence of these substances upon the bone marrow, they were administered in greater concentration than has been hitherto reported. It has been

stated that the administration of thyroid together with a low caloric diet in rats produces leukopenia (54). Therefore, observations were also made on the effect of chloramphenicol on thyroid-induced leukopenia.

II. EXPERIMENTAL STUDIES

A. Studies on the Production of Anaphylaxis by Drugs.

Methods and Materials:

Attempts to produce anaphylaxis in guinea pigs by sensitization with drugs incubated in vitro with autologous blood were made in a series of six experiments (Table II). Employing the technique of Samson and Götz (39) 2.0 ml. of blood were obtained by cardiac puncture and placed in a tube containing 0.5 ml. of 1.33 per cent sodium citrate. To this was added a sufficient volume of 5.0 per cent aqueous solution of amidopyrine, or 0.25 per cent aqueous solution of chloramphenicol so as to provide 100.0 mg./kg. and 50.0 mg./kg. of the drug respectively. The mixture of the drug and autologous blood was then incubated at room temperature for 30 minutes. Thereafter the incubate was reinjected intraperitoneally into the same guinea pig from which the blood was previously withdrawn. A total of three such immunizing injections was made at 6 to 10 day intervals. Following a period of four weeks after the last immunizing dose, the animals were challenged by the intravenous injection of 0.5 ml. of antigen similarly prepared. Seventy guinea pigs were employed in this manner to test the capacity of amidopyrine and chloramphenicol to produce anaphylaxis.

Observations:

Anaphylaxis was observed only in one animal out of 69.

Table II

EXPERIMENTS WITH AMIDOPYRINE AND CHLORAMPHENICOL

ON PRODUCTION OF ANAPHYLAXIS IN GUINEA PIGS

Experiment No.	Immunizing Antigen	Drug Concentration mg./kg./animal	No. of Animals Injected	Anaphylaxis
1	Amidopyrine (Aq) & Blood	100	10	None
2	Chloramphenicol (Aq) & Blood	50	28	1
3	Chloramphenicol (Iso) & Blood	50	10	None
4	Chloramphenicol (Iso) & Blood	50	10	None
5	Chloramphenicol (Iso) & Blood	50	10	None
6	Chloramphenicol (Iso) & Blood	50	9	None

Aq = Aqueous
Iso = Isotonic

Discussion:

In our studies we have not been able to substantiate the work of Samson and Götz. Because of the almost complete absence of an anaphylactic response in these animals by this technic, the method was considered unsuitable as a means of investigating the sensitizing properties of drugs. Differences in the strain of animals or minor differences in technic, may account for our lack of success. The results suggest, under the conditions of our experiments, that antigenic binding of chloramphenicol and amidopyrine did not occur on incubation in vitro with normal guinea pig blood. In spite of this failure, however, there is evidence to suggest that such binding does take place in vivo. Demeshek (32) has demonstrated the occurrence of skin sensitivity in patients sensitive to amidopyrine, utilizing patient's blood and the drug incubated in the ice box for one week. Gell (40) succeeded in producing precipitins in rabbits following the intravenous injections of simple chemical compounds. It is apparent that further studies on this aspect of the problem are warranted.

B. Studies with the Use of Hogologous Bone-Marrow and Blood.

Methods and Materials:

Efforts to produce bone marrow hypoplasia and changes in the formed elements of the blood by sensitizing animals to mixtures of drug with homologous bone marrow or blood were made in a series of 10 experiments (Table III). In these, rabbits and guinea pigs were injected with mixtures of homologous bone marrow and chloramphenicol or one of its congeners. Various injection schedules and different routes of injection were employed as indicated in the table.

Experimental Animals:

Table III

METHODS EMPLOYED IN ATTEMPTS TO PRODUCE BONE MARROW HYPOPLASIA IN EXPERIMENTAL ANIMALS BY SENSITIZATION TO MIXTURES OF HOMOLOGOUS BONE MARROW OR BLOOD IN VARIOUS COMBINATIONS WITH DRUGS

Expt. No.	No. of Animals	Sensitizing antigen	Sensitizing Schedule	Period of Challenge
1	5 Rab.	CAPC bone marrow incubated mixture with FA	6 SC, 4 ml. each at 3 week intervals for 16 weeks	22nd week. 5 ml. IV daily for 5 days
2	5 "	Diamine bone marrow mixture with FA	"	"
3	5 "	Palmitate bone marrow mixture with FA	"	"
4	5 "	CAPC bone marrow azo-protein with FA	4 SC, 4 ml. each at 3 week intervals for 11 weeks	25th week. 5 ml. IV daily for 10 days
5	5 "	Diamine bone marrow azo-protein with FA	"	"
6	5 "	CAPC bone marrow mixture with FA and killed TB	Initial SC 1 ml. then 0.6 ml. ID at 4 sites for 10 weeks	11th week. 5 ml. IV daily for 5 days. Then 40 mg. b.d. orally-5 d
7	20 G.P.	"	10 animals 0.6 ID at 1 week intervals x 3 in 10 animals 1.0 ml. IM & then 0.6 ml. ID at 2 week intervals x 4	30th week 7 animals. 10 mg. b.d. orally for 5 days
8	15 "	CAPC blood mixture (in vivo) with FA & killed TB	0.6 ml. ID every 3 wk. x 3	6th month 7 animals 10 mg. b.d. orally for 5 days
9	15 "	CAPC bone marrow mixture (in vivo) with FA and killed TB	"	—
10	3 "	CAPC liver, spleen and kidney incubated mixtures	15 IP, 1 ml. each 3 X a week x 3	10th week. 50 mg. orally every other day for 10 days

FA, Freund's adjuvant; TB, Tubercle bacilli; SC, subcutaneously; IV, intravenously, IM, intramuscularly; ID, intradermally; IP, intraperitoneally; Rab., rabbits; G.P. guinea pigs, CAPC, chloramphenicol; b.d., twice daily.

New Zealand white rabbits ranging from 1.5 - 2.5 kg. body weight and British short-haired or Hartley strain guinea pigs weighing 400 - 700 gm. were used. The rabbits were housed in groups of five and the guinea pigs were kept in individual cages. Diet for the rabbit consisted of "Purina" rabbit pellets and water ad libitum. Guinea pigs were maintained on Rockland Vitamin C fortified guinea pig diet and water ad libitum.

Immunizing Antigens.

Chloramphenicol* (D (-) threo-2-dichloracetamido-1-p-nitrophenyl-1, 3-propanediol) and two of its congeners (a)* the "Diamine derivative" (D (-) threo-1-p-aminophenyl-2-amino-1, 3 propanediol) and (b)* the palmitic ester of chloramphenicol, hereafter referred to as "diamine" and "palmitate" respectively, were the drugs employed in this series of experiments. They were used in 0.25% aqueous or saline solutions except for the "palmitate" which was in suspension. Homologous bone marrow was obtained from the proximal ends of the long bones of freshly killed animals. The marrow and other selected organs were crushed in a mortar or homogenized in a Waring blendor. The homogenates were then filtered through eight layers of gauze or through a No. 60 fine wire mesh, and suspended in saline. Varying concentrations of these suspensions were employed in different experiments. Equal volumes of the suspensions and of the aqueous solution of the drug selected were then mixed, shaken thoroughly, and incubated at 37° C. for one hour with occasional agitation. At the end of this period, an emulsion was prepared with equal volumes of the incubate and Freund's adjuvant** according

---

*These substances were supplied by Parke, Davis and Company.
**Freund's adjuvant was prepared by mixing 8.5 parts of oil (Bayol F) and 1.5 parts of an emulsifying agent, (Ariace A).

to the technic described by Freund (46).

In certain studies chloramphenicol and its "diamine" congener were diazotized for coupling with bone marrow protein using a technic recommended by Dr. Calvin Bratton of Parke, Davis and Company. To an aliquot of the diazotized material containing approximately 50 mg. of the drug, 2.0 gm. of freshly homogenized bone marrow, suspended in 5.0 ml. of water, were added for coupling. The mixture was left at 5° C. overnight. The following morning, the pH of the mixture was adjusted to 7.0. Equal volumes of the bone marrow azoprotein and Freund's adjuvant were then emulsified in a syringe and the material was ready for injection.

In vivo binding of chloramphenicol to blood or bone marrow was carried out in guinea pigs in the following manner; A donor guinea pig was injected subcutaneously with a 16% solution of chloramphenicol in propylene glycol so that the animal received the drug in an amount of 800 mg./kg. body weight. Blood was obtained by cardiac puncture two hours after the time of injection. To obtain bone marrow, the animal was sacrificed four hours after injection. The blood or the bone marrow was then emulsified in a syringe with Freund's Adjuvant and injected as indicated in Table II.

Challenging Antigens:

For intravenous injection the bone marrow-drug mixture in saline was prepared precisely in the same manner as the corresponding sensitizing antigen, up to but excluding the addition of the adjuvant. At this stage the bone marrow-drug incubate was filtered through eight layers of gauze, or Seitz filtered for certain of the studies. The filtrate was then injected intravenously. Clean but not sterile glassware was used through-

out the procedure. For oral administration 10 to 40 mg. of chloramphenicol were administered twice daily in gelatin capsules.

Studies:

All animals were weighed weekly or twice monthly throughout the period of observation. At appropriate intervals, samples of blood were obtained by cardiac puncture from which total leukocyte, platelet counts and packed cell volumes were determined. The Thomas pipette was employed for all white blood cell enumerations, employing three per cent acetic acid as the diluting fluid, and a dilution of 1:20. Hemoglobin determinations were carried out by the oxyhemoglobin method using the Evelyn photoelectric colorimeter. The volume of packed red cells was determined with the use of the Wintrobe hematocrit (1). Platelets were enumerated by the direct method. Cover-slip preparations of the blood were stained with Wright's stain for differential leukocyte counts.

Observations:

Throughout the period of sensitization and during the entire period of observation thereafter, no significant alterations in the blood were observed in any of the groups studied.

Discussion:

By the technics employed, we have not produced changes in the blood or bone marrow of experimental animals. Among the causes of failure may be the fact that we used as the antigen only simple incubates of tissues, bone marrow or blood with drugs. This method may not have been adequate to secure optimal binding between the drug and blood or bone marrow, despite the claims of Samson and Götz (39). To secure optimal binding by artificial conjugation and to test the antigenicity of drugs conjugated chemically with

blood or bone marrow, we utilized drug-bone marrow azo-protein preparations. Here too, however, coupling of the drug may not have occurred, despite the color changes which indicated adequate diazotization. Moreover, if coupling did take place, the 'blocking' of the reacting NO_2 group of the benzene ring in chloramphenicol may have rendered the compound antigenically inactive.

Tests for the presence of circulating or tissue fixed antibodies were not performed during this study until the production of hypoplastic bone marrow or changes in the blood were assured. Therefore, it cannot be stated whether or not the animals were in any way sensitized to the antigens employed.

Arbitrarily, we worked with a concentration of chloramphenicol which was close to the blood concentration employed therapeutically in man. This level may have been too high to produce a detectible influence on the blood forming organs for it is recognized that an antigen in great condentration may lead to "immunologic paralysis". In this regard recent studies by Morgan et al. (55) have shown that pneumococcal polysaccharide is antigenic in rabbits, and that the previous failures in this respect were due to the use of excess polysaccharide. On the other hand, the amount of material employed may have been insufficiency to produce an adequate titer of antibodies required to mediate the changes for which we were looking.

Assuming that our animals did indeed become sensitized and that specific antibodies to a drug-protein complex were formed, we may have failed to demonstrate changes in the blood or bone marrow because of the operation of an adequate protective mechanism. In this regard, the adrenal cortex may play a role in the homeostatic protection of the animal against the influence of iso-antibodies. Investigations have demonstrated that the administration

of cortisone to intact or adrenalectomized rabbits receiving daily intracutaneous injections of antigen lowered greatly the resulting concentrations of circulating antibodies and inhibited the development of cutaneous sensitivity (50).

In our experiments it is apparent that negative results need not rule out the validity of our working hypothesis. The fact that we have failed to produce bone marrow hypoplasia in experimental animals, using mixtures of homologous bone marrow and blood with drugs does not exclude the possibility that an immunologic mechanism is responsible for hypersensitivity reactions involving hemopoietic tissues in man. Clinically also, it is rare that such hypersensitivity reactions follow the administration of drugs. Even more rare is it to demonstrate a specific immunologic mechanism. There is possibly some mechanism in these individuals that render them particularly susceptible. The whole phenomenon may be one of relatively greater susceptibility to tissue penetration in such patients.

C. Studies with Bone Marrow Antisera.

Methods and Materials:

The influence of bone marrow antisera upon the peripheral blood and bone marrow of experimental animals was examined in three different combinations of species — anti-rabbit bone marrow serum produced in guinea pigs, anti-swine bone marrow serum produced in rabbits and anti-rabbit bone marrow serum produced in swine.

Saline suspensions of fresh, red, bone marrow were emulsified in equal proportion with Freund's adjuvant and injected into animals. For production

of anti-swine bone marrow serum, lyopholized* bone marrow was employed. Varying amounts of bone marrow per animal were injected in different species. Schedules of immunization for each species are recorded in Tables IV, V, VI.

For anti-rabbit bone marrow swine serum, the titer of hemolysin was determined according to the technic described by Kabat and Mayer (57), and was observed to be 1 in 3000. The antiserum also showed the presence of erythrocyte agglutinins by appropriate technics. Similar tests on control normal swine serum were negative.

Two to five ml. of the antiserum were then injected daily by intravenous or intraperitoneal routes for periods up to 56 days. Early death of the recipient animal could be avoided by adjusting the dose of antiserum injected. In most instances, however, this was not required and the animals could tolerate as much as 5 ml. antiserum intravenously every day for prolonged periods.

At the start of the study and again at the height of the blood changes, specimens of the bone marrow were obtained by needle aspiration of the proximal end of the tibia in rabbits and in the sternum in swine. Cover slip smears of blood and bone marrow were stained with Wright's stain. Reticulocytes counts were done at appropriate intervals. All blood studies were made on oxalated specimens obtained by cardiac puncture in rabbits and from the jugular vein in swine.

Observations:

Anti-Rabbit Bone Marrow Guinea Pig Serum.

Changes in the blood of normal rabbits immediately after the injection

---

*Lyopholized swine bone marrow was prepared for us by the courtesy of Armour Laboratories.

Table IV

SCHEDULE OF IMMUNIZATION OF RABBITS TO LYPHOLIZED* SWINE BONE MARROW

Animals Injected	Day of Injection	Vol. of Emulsion Injected/ Animal	Route of Injection	Weight of Bone Marrow Injected/ Animal	Killed TB Injected/ Animal
Group A	1	2.0 ml.	sub. cut.	10.0 mg.	0.50 mg.
15	20	2.0 ml.	sub. cut.	10.0 mg.	0.50 mg.
	30	2.0 ml.	1.4 ml. IM		
			0.6 ml. ID	10.0 mg.	1.00 mg.
	60	0.6 ml.	ID	3.0 mg.	0.30 mg.
	90	1.0 ml.	0.5 sub.cut.		
			0.5 ID	25.0 mg.	0.10 mg.
	120	2.0 ml.	sub. cut.	50.0 mg.	0.40 mg.
Group B	1	2.0 ml.	sub. cut.	10.0 mg.	0.50 mg.
15	7	2.0 ml.	sub. cut.	10.0 mg.	0.25 mg.
	13	2.0 ml.	sub. cut.	10.0 mg.	0.25 mg.
	20	2.0 ml.	sub. cut.	10.0 mg.	0.25 mg.
	61	2.0 ml.	sub. cut.	10.0 mg.	1.00 mg.
	84	0.6 ml.	ID	3.0 mg.	0.30 mg.
	130	1.0 ml.	0.5 ml. S.C.		
			0.5 ml. ID	25.0 mg.	0.10 mg.
	155	2.0 ml.	sub. cut.	50.0 mg.	0.40 mg.
Group C	1	2.9 ml.	1.4 ml. IM		
30			0.6 ml. ID	10.0 mg.	0.50 mg.
	7	0.5 ml.	ID	2.5 mg.	0.25 mg.
	45	1.0 ml.	0.5 ml. S.C.		
			0.5 ml. ID	25.0 mg.	0.10 mg.
	67	2.0 ml.	sub. cut.	50.0 mg.	0.40 mg.

*Lypholized swine marrow was prepared for us by the courtesy of Armour Laboratories.

TB, Tubercule bacilli.
Sub. cut., subcutaneous
S.C., subcutaneous
IM, intramuscularly
ID, intradermally

Table V

SCHEDULE OF IMMUNIZATION OF GUINEA PIGS TO RABBIT BONE MARROW

Animals Injected	Day of Injection	Volume of Emulsion Injected/ Animal	Route of Injection	Weight of Bone Marrow Injected/ Animal	Weight of Killed TB Injected/ Animal
Group A 30	1	2.0 ml.	1.5 ml. IM 0.5 ml. ID	100.0 mg.	1.00 mg.
	7	0.5 ml.	ID	25.0 mg.	0.25 mg.
	42	1.0 ml.	Sub. cut.	10.0 mg.	0.10 mg.
Group B 30	1	1.0 ml.	Sub. cut.	10.0 mg.	0.10 mg.
	22	1.0 ml.	ID	10.0 mg.	0.10 mg.

IM, Intramuscular; ID, Intradermal

Table VI

SCHEDULE OF IMMUNIZATION OF SWINE TO RABBIT BONE MARROW

Animals Injected	Day of Injection	Volume of Emulsion Injected/ Animal	Route of Injection	Weight of Bone Marrow Injected/ Animal	Weight of Killed TB Injected
2 Swine	1	3.0 ml.	1.0 ml. IM 1.0 ml. at 2 sites ID	2.0 gm.	1.0 mg.
	7	3.0 ml.	1.0 ml. at 2 sites ID	2.0 gm.	1.0 mg.
	15	3.0 ml.	1.0 ml. at 2 sites ID	2.0 gm.	1.0 mg.
	35	4.0 ml.	1.0 ml. IM 1.0 ml. at 3 sites ID	2.0 gm.	0.5 mg.
	42	6.0 ml.	2 ml. IM 1.0 ml. at 4 sites ID	3.0 gm.	0.5 mg.
	50	8.0 ml.	2 ml. at ... 2 sites IM 1.0 ml. at 4 sites ID	4.0 gm.	0.5 mg.

sub. cut., subcutaneous

of 5.9 ml. of anti-rabbit bone marrow serum produced in guinea pigs, are recorded in Table VII. It may be observed that the antiserum produced profound leukopenia and thrombocytopenia in these animals. The changes were maximal within 15 to 60 minutes after the injection (Fig. 2) and persisted in certain instances for several hours. When administered every day progressive anemia developed and was maximal on the third day. Thereafter, upon the same dosage schedule there was little change in the degree of anemia. A study of the potency of the antiserum on the 15th day of injection revealed, in some instances, the same profound immediate changes that were observed at the start of the experiment. Administration of normal guinea pig serum in identical doses failed to produce any significant hematologic alterations.

Anti-Swine Bone Marrow Rabbit Serum.

In four swine, the effect of bone marrow rabbit antiserum was almost entirely negligible. Daily doses of as much as 40.0 ml. were administered intravenously and a total of as much as 600 ml. of antiserum was given over a period of 45 days without significant hematologic alterations.

However, a severe "toxic" reaction to the antiserum was observed to occur sporadically in all four swine. This reaction was characterized by flushing, dyspnea, vomiting, urinary and fecal incontinence, cyanosis, ataxia, and syncope. When it occurred, the reaction appeared immediately following the injection of the antiserum and was completely dissipated within 15 minutes in two of the animals. Two other animals died during such a reaction. These reactions were reduced, but not abolished, by first absorbing the antiserum with normal swine blood.

Anti-Rabbit Bone Marrow Swine Serum.

The immediate effects of this antiserum in three normal rabbits were

Table VII

IMMEDIATE EFFECT OF A SINGLE INTRAVENOUS INJECTION OF ANTI-RABBIT BONE MARROW GUINEA PIG SERUM ON THE BLOOD OF RABBITS

Rabbit No.	Injection No.	Time	Leukocytes x 10^3 per cu.mm.	P.M.N.'s	M.N.C.	V.P.R.C. per ml.	Platelets x 10^3 per cu. mm.
1	1	Before	7.6	4,000	3,600	38.5	510
		After*	3.8	950	2,900	36.0	170
	2	Before	7.1	3,550	3,550	38.0	320
		After*	2.4	325	2,125	35.5	140
2	1	Before	10.3	2,225	8,100	36.0	-
		After*	2.9	125	2,825	35.0	-
3		Before	9.2	2,990	6,300	39.5	620
		After*	2.7	50	2,650	20.0	190
4.	1	Before	10.7	5,900	4,800	39.5	-
		After**	3.1	400	2,750	40.0	-

*After 60 minutes.
**After 30 minutes.
P.M.N., Polymorphonuclear leukocytes including basophils and eosinophils.
M.N.S., Mononuclear cells including monocytes, lymphocytes and plasma cells.
V.P.R.C., Volume of packed red cells.

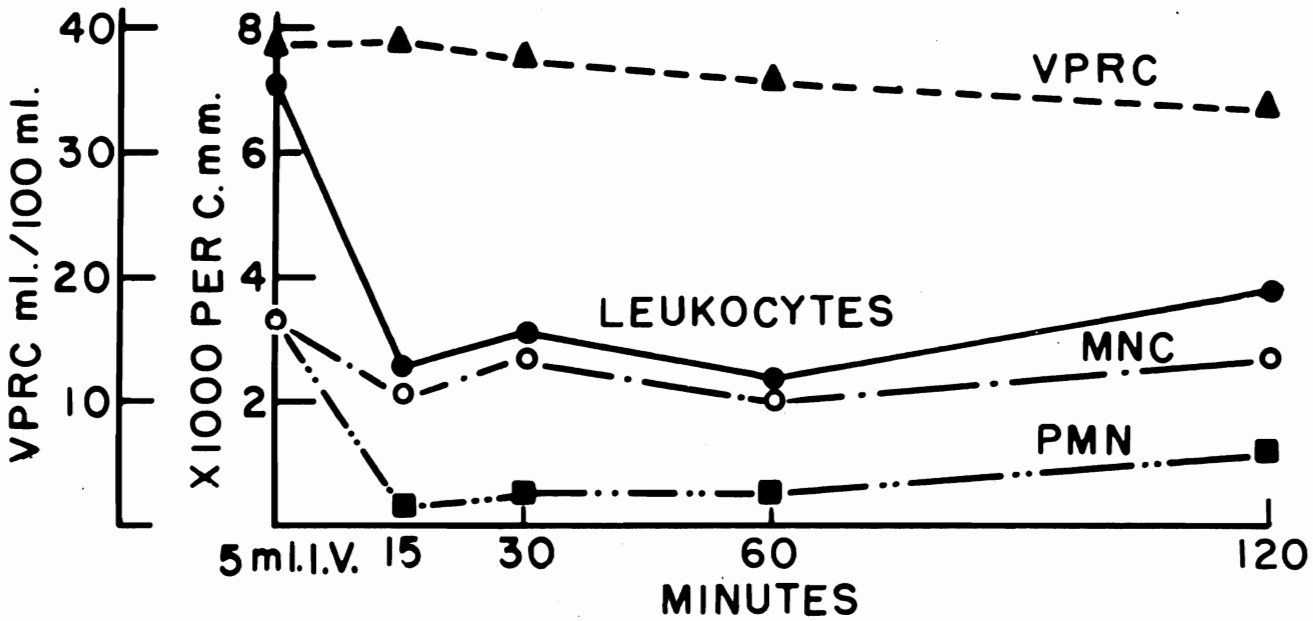


Figure 2. Immediate effects of a single intravenous injection of anti-rabbit bone marrow guinea pig serum on the blood of a rabbit.

qualitatively identical to those observed in rabbits injected with guinea pig antiserum. However, similar changes were also observed immediately following the intravenous injection of normal swine serum as well (Table VIII). When administered daily over long periods of time, however, the animals receiving antiserum became progressively anemic (Table IX), whereas no significant changes in the hemoglobin content were observed in the group that received normal swine serum.

At the height of the anemia, blood smears showed micro-spherocytosis, marked anisocytosis and an increase in the number of reticulocytes. In addition, many basophilic and nucleated erythrocytes were present (Fig. 3). Neither hemorrhages nor jaundice was observed. The total leukocyte counts showed a moderate increase due mainly to an increase in the number of mononuclear cells. Polymorphonuclear cells either decreased or were unchanged. There was a progressive fall in the volume of packed red cells. However, when the schedule of injection was changed from 5 ml. daily to 5 ml. biweekly, an increase was observed in the level of packed red cells (Fig. 4).

Of the five animals that were injected daily with the antiserum, three died of anemia. Each of these animals had received a total of 70, 80, and 200 ml. of the immune serum over a period of 14, 16, and 40 days, respectively. On post mortem, the internal viscera looked pale and anemic. The liver showed a nutmeg appearance in all the cases. The bone marrow in each case was red and hyperplastic by gross examination. Microscopic examination of the bone marrow smears in these animals at the height of anemia, and a few days preceding death, showed it to be hypercellular with an increased number of immature erythrocytes, many of them in mitosis. No changes were observed in granulocytes.

Table VIII

IMMEDIATE CHANGES IN THE BLOOD OF RABBITS FOLLOWING SINGLE INTRAVENOUS INJECTION OF NORMAL SWINE SERUM AND ANTI-RABBIT BONE MARROW SWINE SERUM

Material Animal	Injection	Normal Swine Serum Leukocytes x 10^3/ c.mm	V.P.R.C. per ml.	Platelets x 10^3/ c.mm	Rabbit bone marrow swine antiserum Leukocytes x 10^3/ c.mm	V.P.R.C. per ml.	Platelets x 10^3/ c.mm
1	Before	8.9	43.5	250	12.4	39.0	370
	After*	2.3	41.5	30	1.6	35.0	140
2	Before	9.5	40.0	450	7.0	40.5	390
	After*	3.3	39.0	160	1.7	34.0	90
3	Before	7.6	43.5	560	11.6	40.5	650
	After*	2.7	42.5	240	0.3	38.5	200

*within 60 minutes.

Table IX

EFFECT OF PROLONGED INTRAVENOUS INJECTIONS OF ANTI-RABBIT BONE MARROW SWINE SERUM ON THE BLOOD OF RABBITS

Rabbit No.	Injection Dose	Time	Leukocytes x 10^3 per cu.mm	P.M.N.'s	M.N.C.	V.P.R.C. per ml.	Platelets x 10^3 per cu.mm
1.	5 ml.	Before	15.0	3,600	11,400	36.0	450
		After -6 days	18.2	2,200	16,000	28.5	500
		After -15 days	15.5	4,320	10,180	21.0	680
2.	5 ml.	Before	16.0	6,720	8,280	42.0	550
		After -10 days	20.2	2,000	18,200	17.5	150
		After +16 days	14.2	1,200	13,000	23.0	290
3.	5 ml.	Before	12.3	4,675	7,625	42.0	370
		After -3 days	18.1	2,175	15,925	34.0	85
		After -10 days	16.3	1,800	14,500	15.0	540
4.	5 ml.	Before	7.5	2,700	4,800	44.0	500
		After -3 days	8.6	1,000	7,600	37.5	600
		After -11 days	22.0	8,600	13,500	20.5	230
5.	5 ml.	Before	7.4	4,292	3,208	40.0	400
		After -7 days	12.7	1,900	10,800	18.5	650
		After -56 days	9.0	—	—	21.0	290

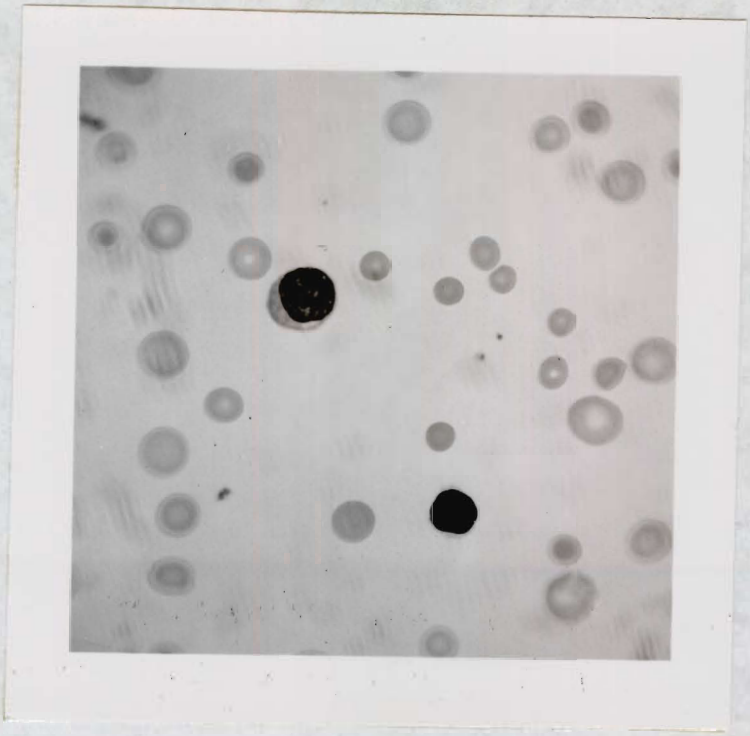


Figure 3. Blood smear of a rabbit injected with anti-rabbit bone marrow swine serum. Note marked anisocytosis, spherocytosis and a nucleated red cell.

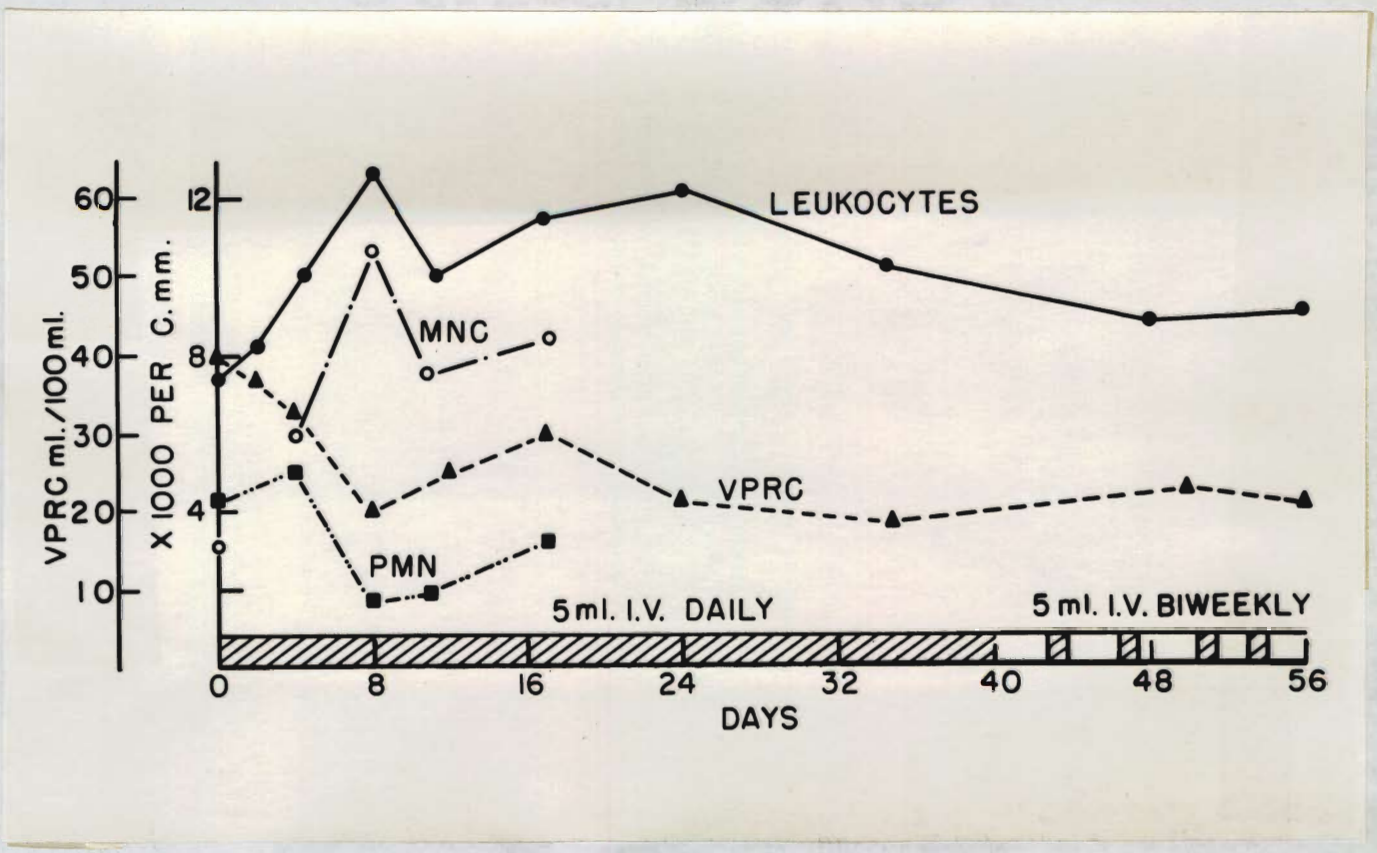


Figure 4. Changes in the blood of a rabbit following prolonged intravenous injections of anti-rabbit bone marrow swine serum.

Discussion:

The production of aplasia of the bone marrow by means of antibodies, regardless of their sources, would be a valuable means for the study of the physiology of the blood forming organs. Bunting (58) in 1904 reported the occurrence of significant bone marrow alterations in rabbits following injections of anti-rabbit bone marrow goose serum. He referred to the bone marrow picture as one of "entire depletion from the marrow of mature elements, and injury and destruction of the immature cells". More recently Bjorkland and Hallstrom (49) have claimed to produce aplasia of the bone marrow in rabbits by the use of anti-rabbit bone marrow horse serum. In our studies, however, despite the administration of as much as 200 ml. of antiserum over a period of 40 days the bone marrow was still normal or hypercellular.

Despite this failure to produce aplasia of the bone marrow, the antisera employed none-the-less did contain antibodies to all the formed elements of the blood. Following daily injections of the antiserum, profound anemia developed. The anemia progressively increased as the daily injections were continued. It was hemolytic, and decidedly not hypoplastic in character. Moreover, it was reversible to some extent when injection of antiserum was reduced from daily to bi-weekly intervals (Fig. 4). A similar type of anemia has been previously reported by Dameshek (59).

Leukopenia and thrombocytopenia, in addition, was observed in the rabbits immediately following the intravenous injection of the antiserum. This effect, however, was only transitory in contrast to the persistent and even increasing anemia. The leukopenic effect appeared to be more prominently directed against the polymorphonuclear cells than the lymphocytes, although these too were reduced in numbers in several instances. Shortly after the

beginning of the injections, however, the number of circulating leukocytes remained normal or increased. This occurred in spite of the fact that the antiserum still retained its ability to produce a promptly occurring leukopenia even after 15 days of injection. From these observations it appears possible that in response to the anemia the bone marrow increased its productivity to a remarkable degree, producing white cells in addition, at such a pace as to maintain their normal numbers in the circulation.

Alterations in the number of platelets were too variable to allow anyq reliable conclusions to be derived from the observations made. Suffice it to say that purpura was never observed throughout the study.

Thus, it is evident that elements in the bone marrow of rabbits have produced antibodies in the serum of swine and guinea pigs. These antibodies admittedly may be due to the mature elements of the blood present in the bone marrow, or to their precursors, or to both. Bracco et al. (50) utilized the separated erythroid, myeloid and platelet precursors in the bone marrow of rats to produce specific antiserum in rabbits. Subsequently, the administration of the rabbit antiserum into rats was followed by hypoplasia of the erythrocytic elements in the bone marrow and anemia in the blood. Similarly, antiserum to myeloid precursor cells of the bone marrow was utilized by Bracco to produce myeloid hypoplasia in the bone marrow and leukopenia in the blood. Recently Moeschlin (52) has produced hypoplasia of the myeloid series of the bone marrow in guinea pigs by the use of antiserum to guinea pig leukocytes produced in the rabbit. Thus, it is suggested that the bone marrow may be profoundly altered following injections of antisera produced against one or more of its cellular constituents or against one or more of the formed elements of the blood. However, the

production of complete aplasia of the bone marrow together with the classical picture of aplastic anemia by the use of antiserum has not as yet been reported.

Following these studies (52) Moeschlin has reactivated the hypothesis that an accelerated removal of the various formed elements of the blood from the circulation by means of an immunologic mechanism may lead to a "replacement" hyperplasia of the bone marrow. Under a prolonged stress of this sort the bone marrow may eventually become depleted and finally exhausted, revealing at this time a reduction or complete absence of the cellular element involved. Moeschlin has extended this concept to apply also to drug-induced hypoplastic reactions of the bone marrow. There is little or no experimental evidence for the concept that this mechanism applies in instances of drug sensitivity other than by analogy. Indeed, many cases of hemolytic syndromes can be cited which go on for years with a markedly increased activity of the bone marrow without the development of erythroid aplasia.

In regard to the pancytopenic aspect of normal swine serum on the blood of rabbits, it appears that species difference exists in this respect. Since the demonstration of heterophile antigens by Forssman (60) such reactions have been reported in a number of species (61). It has been shown (62) that normal human, dog and sheep plasma contain anti-rabbit platelet and erythrocyte factors which produce thrombocytopenia and anemia in rabbits. Such reactions are also known to occur between Macacus rhesus monkey and swine (47). The significance of any studies with heterologous antiserum must therefore be properly interpreted in the light of the influence of such heterophile antibodies.

D. Studies on the Toxic Effects of Chloramphenicol.

Methods and Materials:

Studies of the changes in the blood and the rate of growth of young weanling rats were made in a series of six nutritional experiments. Chloramphenicol or its "diamine" congener were fed in concentrations of 1, 2, 3, and 5 per cent of the diet, as shown in Table X. The control group of animals were maintained on normal basal diet ad libitum. In later studies a second control group was pair-fed with the test group of animals to rule out the influence of malnutrition as such.

Palmer et al. have shown (54) that the administration of thyroid extract in rats maintained on a diet characterized by a restricted caloric intake is followed by a retarded rate of growth and leukopenia. We maintained rats on a similar diet (containing 0.6% of dessicated thyroid*) to which was added chloramphenicol (2% of the diet).

Animals:

Approximately 175 albino male rats of Sprague-Dawley strain, weighing 40 - 250 grams, were used. They were housed separately in wire cages.

Diet:

A basal diet of the following percentage composition was used: casein (Sheffield hot alcohol extracted), 20; sucrose, 64; lard, 11; salt mix** 5.

---

*Dessicated thyroid: U.S.P., Abbott -- List 9839

**The salt mixture was of the following percentage composition: NaCl, 13.5; $MgCO_3$, 8.5; KH_2PO_4, 5.7; $CaHPO_4$, 46.3; KCl, 6.7; KI, 0.227; $CaCO_3$, 16.9; $Fe_4(P_2O_7)$, 2.1; $CuSO_4$, 0.022; $MnCl_2$, 0.019; ZnO, 0.016; $CoCO_3$, 0.016.

Table X

EXPERIMENTS ON TOXIC EFFECT OF CHLORAMPHENICOL IN WEANLING RATS

Expt. No.	Group	Animals Employed	
1	1	10	Basal Diet
	2	10	Basal diet containing 1% diamine
2	1	12	Basal Diet
	2	12	Basal diet containing 3% diamine
	3	12	Basal diet containing 3% chloramphenicol
3	1	12	Basal Diet
	2	12	Basal diet containing 3% chloramphenicol
	3	12	Basal diet pair-fed with Group 2
4*	1	36	Basal diet with 3% chloramphenicol
5	1	10	Basal diet with 5% chloramphenicol
	2	10	Basal diet pair-fed with Group 2
6	1	8	Basal diet with 0.6% desiccated thyroid powder and 2% chloramphenicol
	2	8	Basal diet with 0.6% desiccated thyroid powder

*Only in Experiment No. 4 were adult rats employed.

vitamins were added to this diet as follows (amount per kilogram of diet): thiamin hydrochloride, 12.5 mg.; riboflavin, 6.0 mg.; nicotinic acid, 60.0 mg.; pyridoxine hydrochloride, 10.0 mg.; calcium pantothenate, 25.0 mg.; inositol, 5.0 mg.; para-amino-benzoic acid, 5.0 mg.; pteroylglutamic acid, 15.0 mg.; choline chloride, 2.54 gm.; vitamin A, 23,000 units; vitamin D, 4,250 units; vitamin E, 7.6 mg.; vitamin K, 7.6 mg.

Studies:

The animals were weighed every week. All hematologic determinations were made from free-flowing tail-vein blood at weekly intervals or more often if indicated. The methods for the determination of hemoglobin and the enumeration of the leukocytes have been previously described (p.18).

Observations:

On a diet containing 1% of "diamine" a retardation in the growth of the animals was observed in the third week. Upon withdrawal of the drug from the diet in the fifth week, the rate of growth once again accelerated to equal the rate of growth of the animals in the control group (Fig. 5). No singificant alterations were seen in the leukocyte count.

In another experiment, three groups of 12 animals each were used. The control group received the normal basal diet ad libitum and the other two groups were kept on a diet containing 3% of "diamine" and chloramphenicol, respectively. Of greatest interest are the changes which occurred in the chloramphenicol-fed animals. In this group, retardation in the rate of growth was first observed in the second week. (Fig. 6). These animals developed severe toxic symptoms manifested by anorexia, abdominal distension, diarrhea and vomiting. Their fur was poor, discolored brown, and was scanty (Fig. 7). In the "diamine" group, no changes were observed other than those

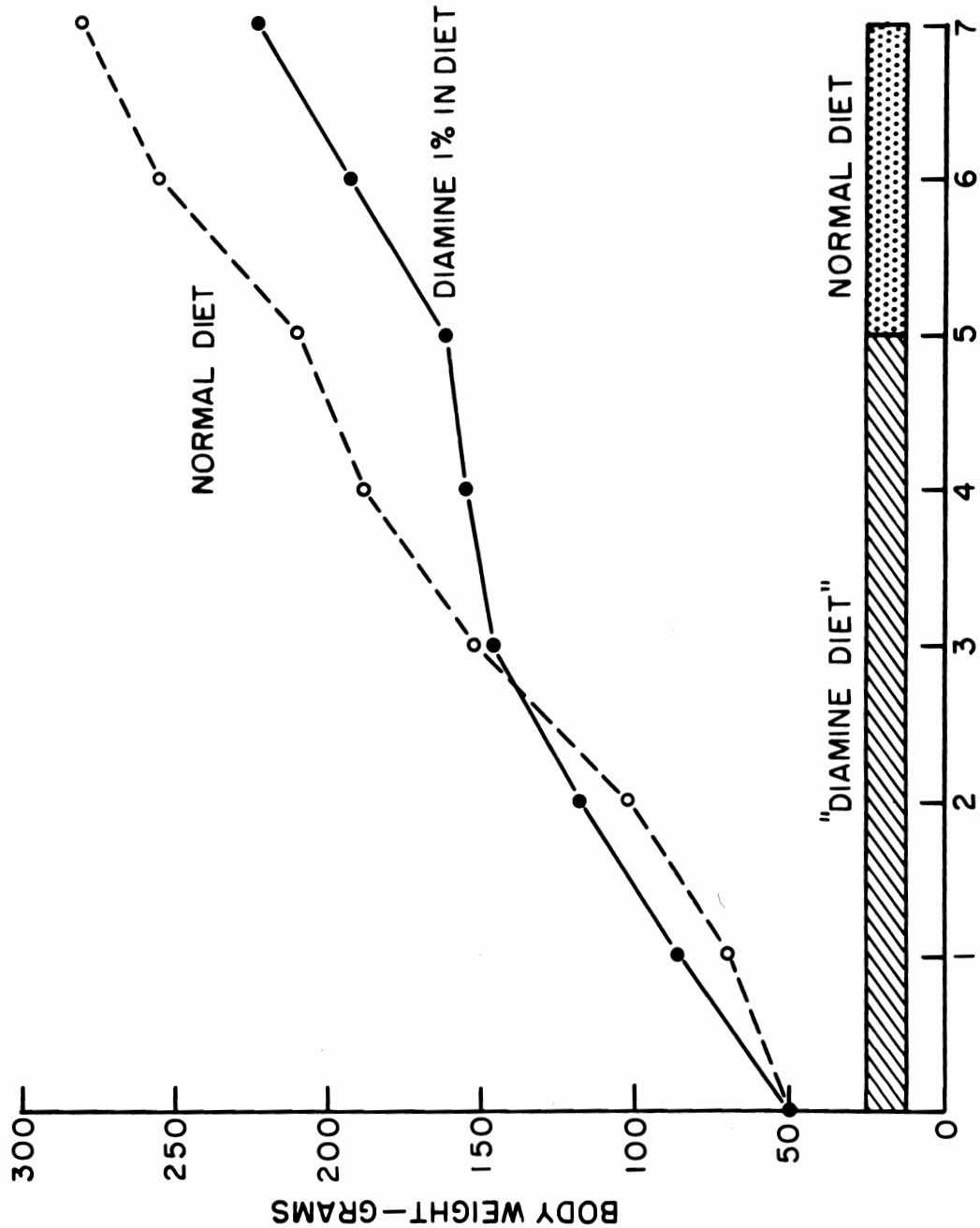


Figure 5. Change in the rate of growth of weanling rats fed 1% "diamine" in diet.

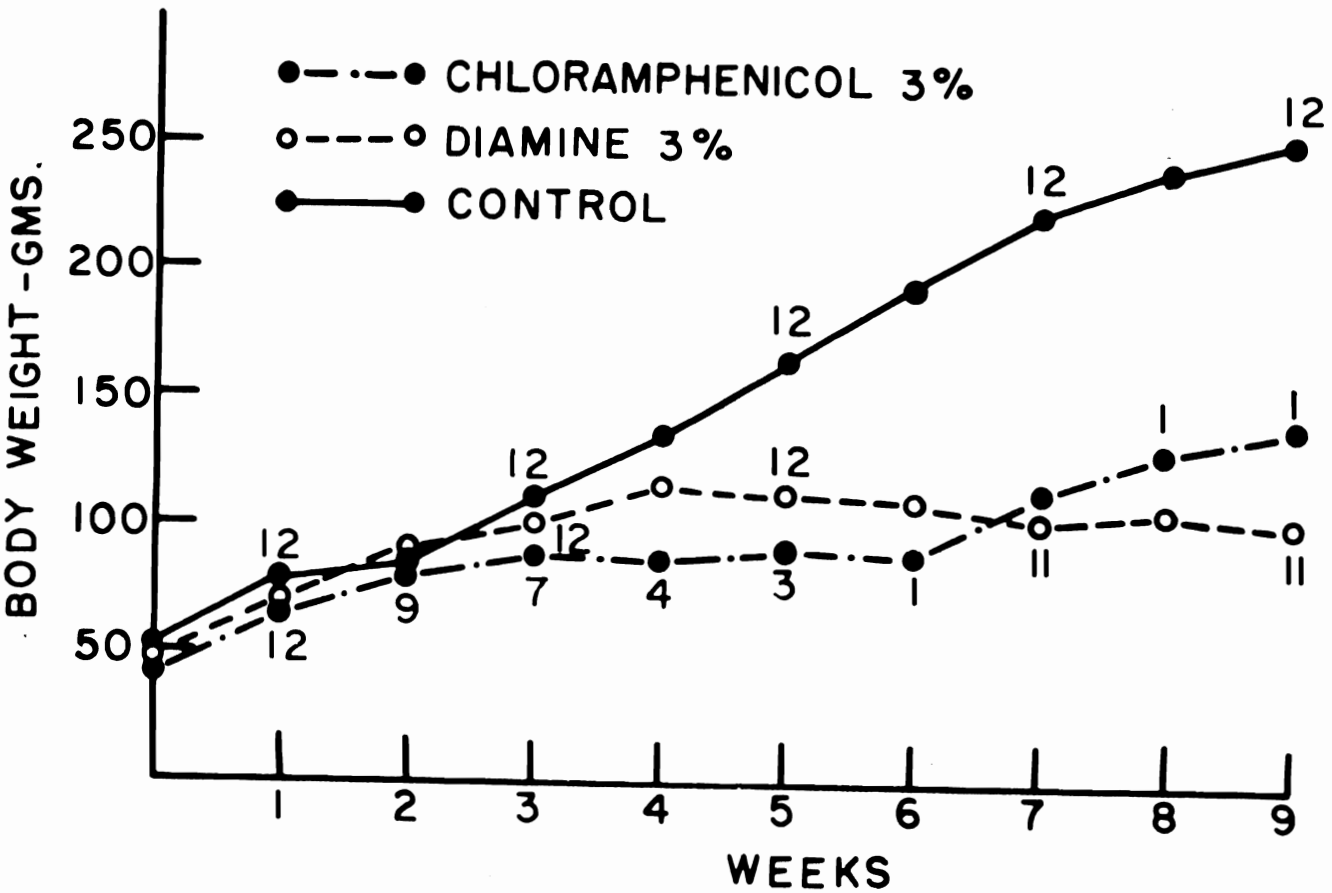


Figure 6. Change in the rate of growth of weanling rats fed "diamine" and chloramphenicol 3% in diet. The numbers at each point represent the surviving animals at that time.

Figure 7. (A) Rat fed normal diet.

(B) Rat fed 3% chloramphenicol in diet.

previously described.

The occurrence of death in the two groups is compared in Fig. 8 A. It may be noted that by the end of six weeks, 90% of the animals receiving chloramphenicol had died, whereas there was no death in the "diamine" group at that time. A reduction in the leukocyte count of chloramphenicol-fed animals was also observed as compared with those in the other groups (Fig. 8 B).

The significance of the alterations in the total leukocyte count of the animals fed chloramphenicol could not be evaluated because of the occurrence of marked anorexia and diarrhea. In a subsequent experiment, therefore, the group of animals fed 3% chloramphenicol in the diet was accompanied by another group of pair-fed animals which received normal diet. By this means the caloric intake in the two groups was approximately the same.

Fig. 9 A shows that the retardation in the rate of growth of the animals in the two groups was approximately the same. However, it may be noted (Fig. 9 B) that the death rate in the test group was again significantly higher than in the control. There were no significant alterations between the average leukocyte counts of the two groups, although it was observed that a number of animals in the test group showed a profound terminal leukopenia (Figs. 10 A, and 10 B).

With an increase in the concentration of chloramphenicol to 5% of the diet, the onset of symptoms was very rapid. The animals failed to grow and, within 1 - 2 days, became extremely malnourished, emaciated and died. The pair-fed partners lived almost twice as long and were distinctly in better shape (Table XI). However, the terminal leukocyte count in both the groups was profoundly reduced.

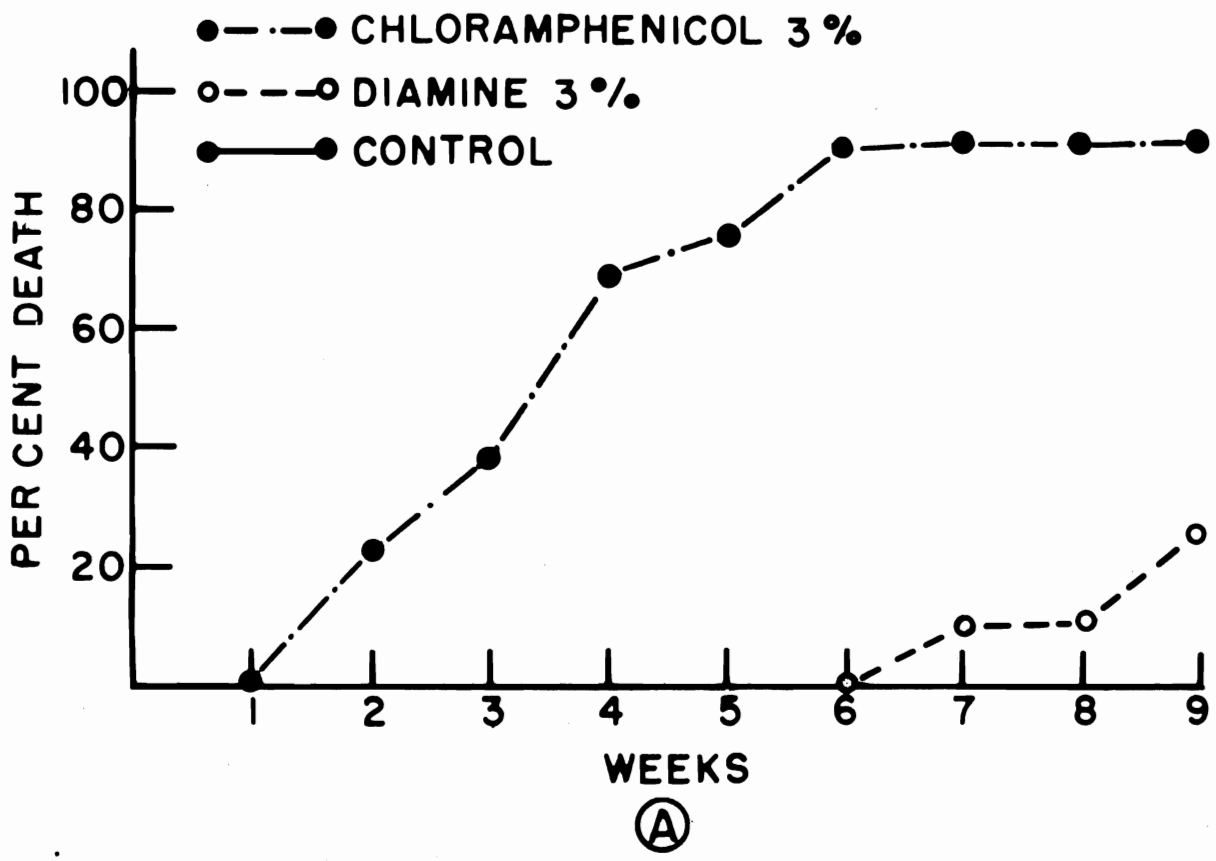


Figure 8A. Rate of death of rats fed 3% diamine and chloramphenicol in diet. There were no deaths in the control group.

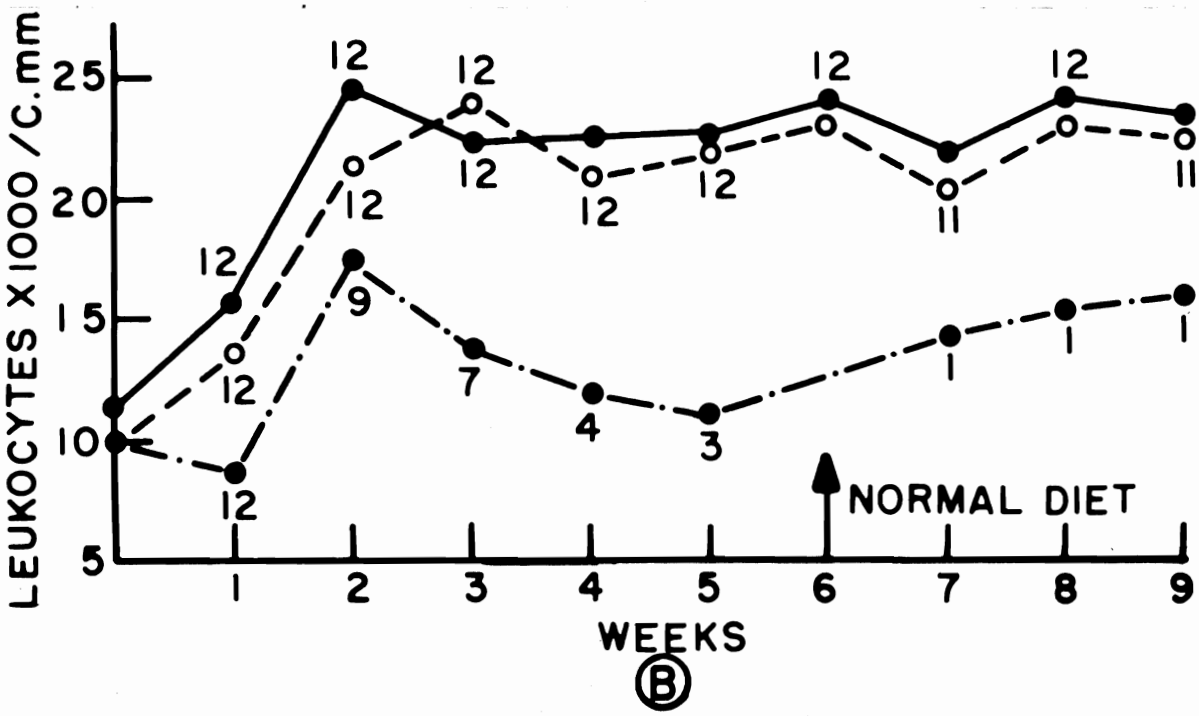


Figure 88. Changes in total leukocytes of rats fed diamine and chloramphenicol 3% in diet. The number at each point represents the surviving animals at that time.

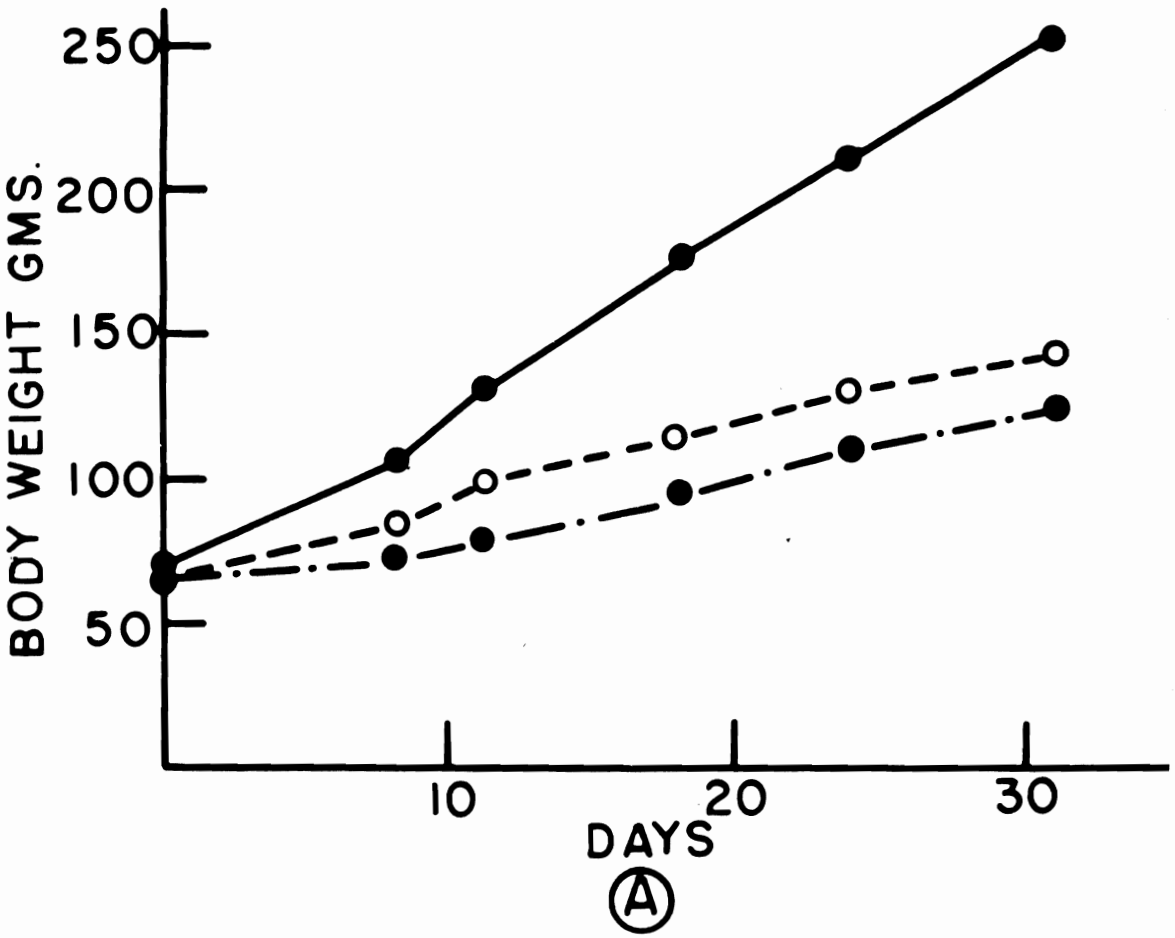


Figure 9A. Changes in body weight of rats fed chloramphenicol 3% in diet and of control pair-fed.

For Legend see Figure 9B

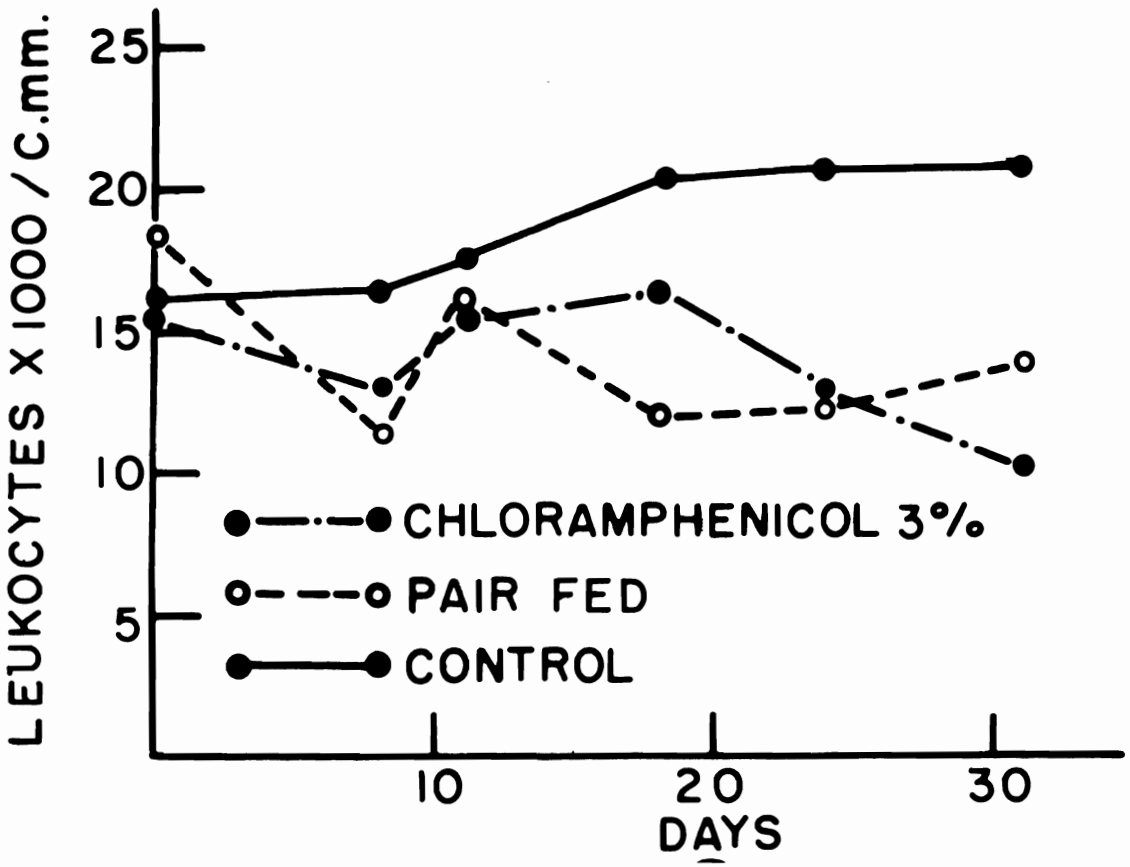


Figure 10A. Changes in leukocytes of rats fed chloramphenicol 3% of diet and of control pair-fed.

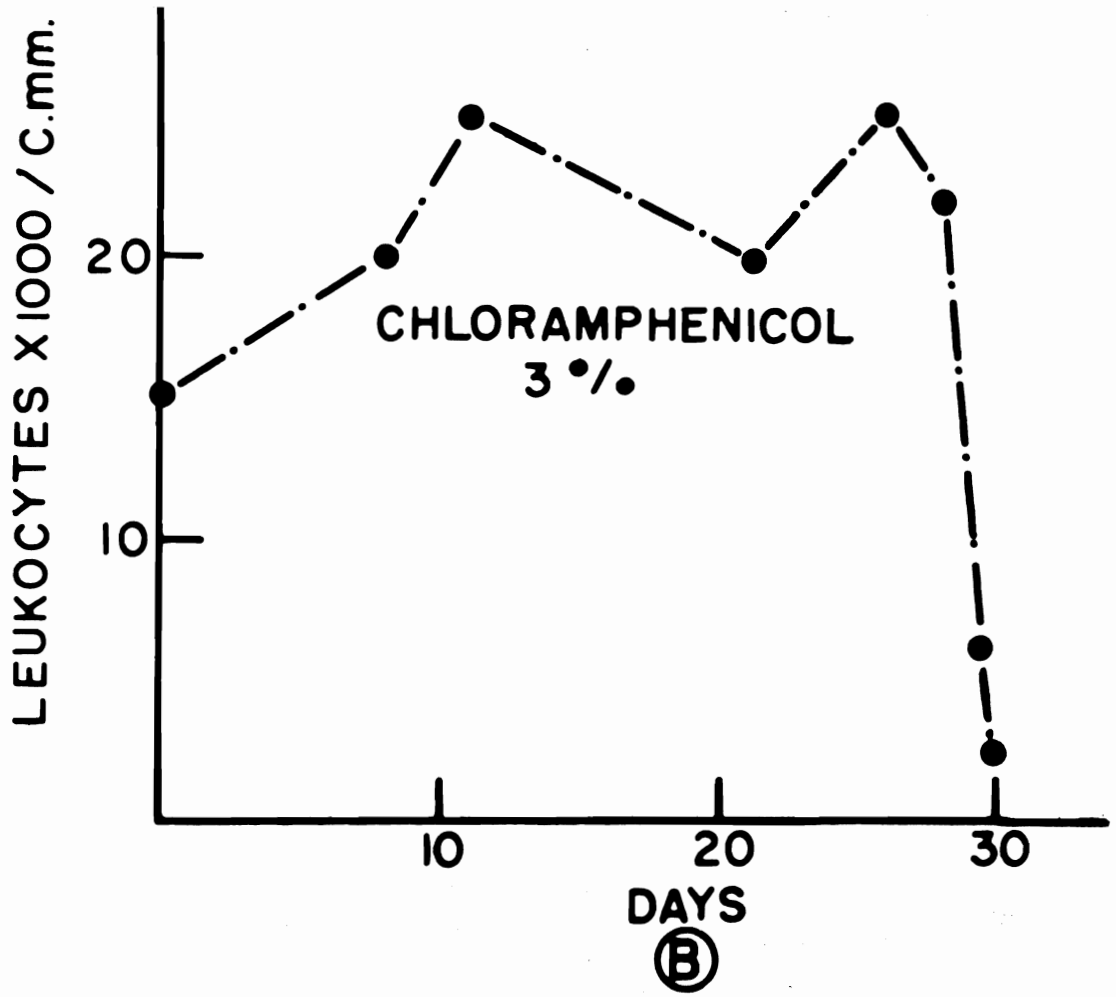


Figure 108. Terminal leukopenia in a rat fed 3% chloramphenicol in diet.

Table XI

CHANGES IN TOTAL LEUKOCYTE COUNT, BODY WEIGHT AND SURVIVAL OF WEANLING RATS FED CHLORAMPHENICOL 5% IN DIET.

Days	Animals Surviving		Weight (Gms.)		Leukocytes x 10^3 per cu.mm.	
	Pair-fed (10)	Chloramphenicol 5% (10)	Pair-fed (10)	Chloramphenicol 5% (10)	Pair-fed (10)	Chloramphenicol 5% (10)
1	10	10	54.0	55.4	15.40	15.90
3	10	10	54.0	45.1	14.80	4.30
4	10	3	53.3	43.0	13.80	1.40
5	10	0	48.8	—	8.80	—
6	10	0	49.1	—	6.20	—
7	4	0	45.0	—	2.60	—
8	2	0	45.0	—	2.10	—

In a study of the influence of chloramphenicol upon the rate of growth and leukocyte counts of weanling rats fed dessicated thyroid, no significant differences were observed between the test group and a control group receiving a similar but chloramphenicol-free diet.

Discussion:

That the toxic signs other than death herein described for weanling rats fed chloramphenicol is due to the effect of the drug, is clear from the observation that such signs failed to developed in the pair-fed control group. With increasing concentration of the antibiotic in the diet, moreover, the onset of these symptoms was more rapid and more pronounced. It has been stated (3) that oral administration of chloramphenicol to young rats leads to anorexia and weight loss. Occurrence of abdominal distension and diarrhea has been reported (4). It is reasonable to suppose that these manifestations are mainly due to an alteration in the bacterial flora of the alimentary tract under the influence of chloramphenicol. That the generalized poor state of these animals and the loss of fur is a direct effect of the drug, or due to some other mechanism has not been clearly ascertained. It is possible that chloramphenicol deprives the organism of some factor essential for its normal well-being, and that young animals are particularly susceptible to this mechanism, since we failed to observe similar changes in adult rats fed chloramphenicol in the amount of 3% of the diet (Table XII). In this respect the "diamine" is relatively safe. This is also indicated by the difference in the influence that these two substances have on the rate of survival of the animals.

The mechanism of action of chloramphenicol as an antibiotic or even as a toxic agent as herein rpported, is not clear. Because of the close

Table XII

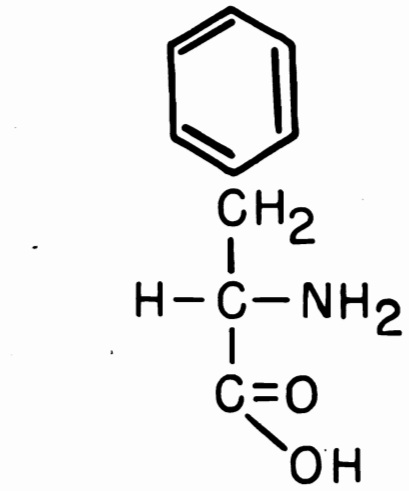
CHANGES IN BODY WEIGHT AND LEUKOCYTE COUNT OF ADULT RATS FED CHLORAMPHENICOL 3% IN DIET

Days	Weight (Gms.) (3%)	Leukocytes x 10^3 per cu. mm. (3%)
1	254.0	18.70
2	256.0	18.40
3	255.0	19.80
5	250.0	18.21
8	242.0	17.40
15	254.0	20.32

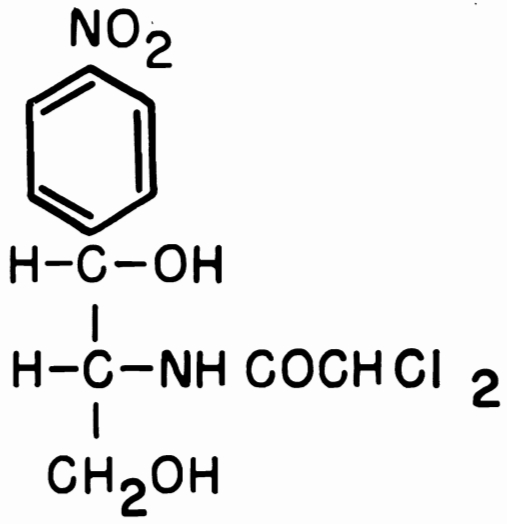
resemblance of chloramphenicol to phenylalanine (Fig. 11), Wooley (29) compared the effect of this antibiotic with the effect that alterations of the structure of pehnylalanine had upon the growth of E. coli. Chloramphenicol, in the presence of small concentrations of pehnylalanine inhibits the growth of E. coli. This is not true for larger concentrations of the amino acid. The replacement of an hydroxyl group for a hydrogen atom in the beta position of phenylalanine is followed by complete inhibition of the growth of the bacteria. Only four substitutions are required of chloramphenicol to arrive precisely at this structure. The presence of three of the four alterations leads to the formation of only a weak inhibitory agent for growth of E. coli. This effect may be represented by the influence of the "diamine" used in several of the studies herein reported. The actual pathway for the degradation of chloramphenicol in the bacterial system using E. coli is depicted in Fig. 12. These observations may explain why, in our studies on weanling rats, chloramphenicol appeared to be far more toxic than was its diamine derivative.

The significance of these observations is not as yet clear. The failure of 3% of chloramphenicol in the diet to produce significant leukocyte alterations compared to the profound terminal leukopenia observed under the influence of 5% of chloramphenicol in the diet cannot be explained, unless it be that the terminal leukopenia is a non-specific phenomenon unrelated to the toxicity of chloramphenicol. The question may be raised, moreover, as to whether chloramphenicol does actually inhibit the activity of phenylalanine. Wooley's studies revealed that this was in part true. The inhibition was not a simple competitive one, however.

A clear relationship to tryptophene has been shown for chloramephenicol



PHENYLALANINE



CHLORAMPHENICOL

Figure 11. Structural formulae of chloramphenicol and phenylalanine.

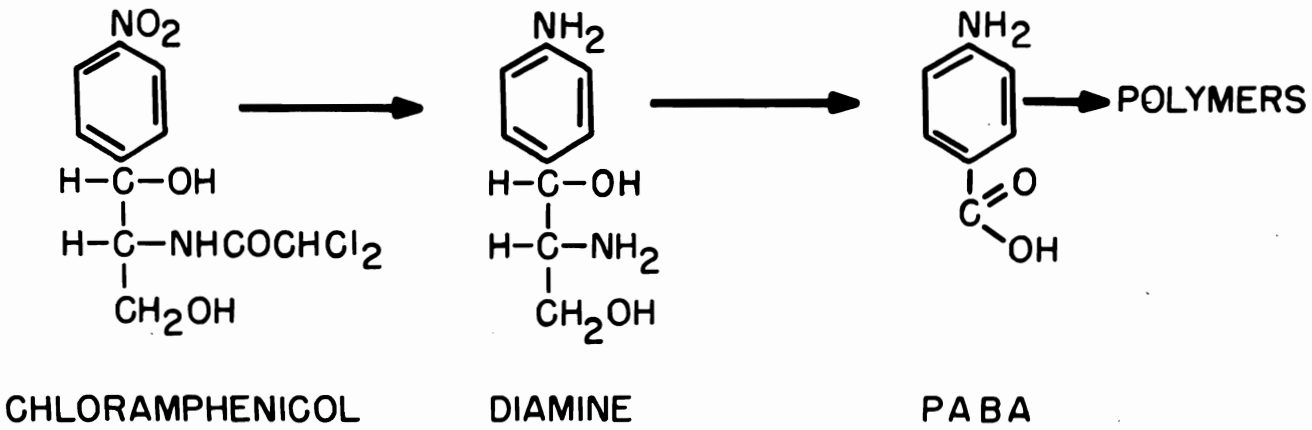


Figure 12. Pathway for the degradation of chloramphenicol in bacterial system using E. coli. PABA, para-aminobenzoic acid.

by Bergmann (31). In this study, chloramphenicol was shown to inhibit the synthesis of tryptophane from anthranilic acid. With the addition of indole or tryptophane the growth of the bacteria was resumed. The addition of phenylalanine or tyrosine was less effective.

What relationship to drug sensitivity these observations of the toxic effect of chloramphenicol in weanling rats may have is not clear, for in animals and humans phenylalanine and tryptophane are essential amino acids and thus, not synthesized in vivo. It may be however, that chloramphenicol may act to inhibit the utilization of phenylalanine or tryptophane in certain individuals. In these individuals in whom "sensitivity" to chloramphenicol has occurred, the drug may be degraded in an unusual fashion. The abnormal metabolite may attain a sufficiently high concentration to inhibit the activity of phenylalanine or tryptophane or both. This may be the mechanism in those individuals in whom the reactions appears to be toxic in nature and reversible. However, in great many instances the reaction is irreversible despite the removal of the drug. The influence of a toxic derivative of chloramphenicol is very unlikely in these instances.

III. RECOMMENDATIONS FOR FUTURE STUDY

A number of questions have to be answered before a proper understanding may be had of the mechanism which mediates the abnormal reactions of drug sensitivity. Although there is experimental evidence to suggest that certain drugs act in an immunologic fashion to produce abnormal blood reactions, whether or not the administered drug or one of its metabolites links with body tissues and becomes antigenic is not known. The possibility that drugs may conjugate with cells elsewhere than in the bone marrow, should be investigated. Use of organ homogenates from an individual known to have died from drug hypersensitivity may be valuable in this respect. The possibility that a toxic drug can penetrate the cell barrier under certain special circumstances and that the resulting sensitivity depends on this selective permeation is another field for investigation. It still remains to be determined whether the drug or the "drug tissue complex" acts on the formed elements of the blood in the circulation or on their precursors in the bone marrow. In the investigation of this problem studies may be undertaken on the uptake of drugs by immature cells of the bone marrow as observed in bone marrow cultures. The capacity of drugs to penetrate the red cell membrane in various normal and abnormal individuals may also be profitably investigated.

Further studies regarding the features which regulate normal cell division, and differentiation and maturation may lead to a better understanding of drug-induced hemocytopenic reactions.

Recent studies suggest that with the use of a single type of cell separated from the bone marrow more potent antisera to the cellular elements of bone marrow may be obtained. Anti-erythrocytic and anti-

leukocytic sera have been claimed to produce selective hypoplasia of the erythroid and myeloid elements of the bone marrow, respectively. Injections of these sera combined may be the method to induce aplastic changes in the bone marrow.

The suggestion of Kracke that the 'benzamine ' ring in drugs is potentially toxic to bone marrow needs substantiation.

IV. SUMMARY

1. Attempts have been made to produce anaphylaxis in guinea pigs by the injection of mixtures of drugs and autologous blood. These attempts were unsuccessful.

2. Efforts were made to produce degenerative changes in the bone marrow and alterations in the blood by injection of homologous bone marrow in rabbits and guinea pigs. These efforts were unsuccessful.

3. The influence of bone marrow antisera upon the blood and bone marrow was observed in rabbits, guinea pigs and swine. In rabbits a prompt and profound pancytopenia occurred after the injection of swine and guinea pig antiserum. With the continuous intravenous administration of bone marrow antiserum in rabbits, only a profound hemolytic anemia was observed, the leukocytes and platelets being normal in the blood. The bone marrow revealed erythroid hyperplasia.

4. The influence of chloramphenicol and its "diamine" congener upon the rate of growth and leukocyte count of weanling rats was observed. The development of anorexia, coarse and discolored hair, abdominal distension and diarrhea appeared to be due to the influence of the drug. Significant alterations in the leukocytes, however, did not appear to occur in relation to the administration of the drug.

BIBLIOGRAPHY

1. Wintrobe, M.M., Clinical Hematology, Lea and Febiger, Philadelphia, 1952.
2. Plum, F., Clinical and Experimental Investigations in Agranulocytosis. Lewis and Co., London. 1937, p. 410.
3. Madison, F.W. and Squier, T.L., J.A.M.A., 102, 755, 1936.
4. Loewy, F.E., Lancet, 1, 845, 1934.
5. Ackroyd, J.F., Progress in Allergy, 3, 531, 1952.
6. Falconer, E.H., and Epstein, N.N., Arch. Int. Med., 65, 122, 1940.
7. Wraith, D.G., Proc. Royal. Soc. Med., 44, 922, 1951.
8. Larson, R.K., Blood, 8, 16, 1953.
9. Watkins, E., and Weld, M.E., J.A.M.A., 147, 755, 1951.
10. Lewis, C.N., Putnam, L.E., Hendricks, F.D., Kerlan, I., and Welch, H., Antibiotics and Chemotherapy, 2, 201, 1952.
11. Racinos, J.R.A., Ross, S., Olshaker, B., and Twible, E., New England J. Med., 241, 733, 1949.
12. Hodgkinson, R., Lancet, 1, 283, 1954.
13. Heck, F.J., Post Graduate Medicine, 13, 105, 1953.
14. Osgood, E.E., Ann. Int. Med., 39, 1173, 1953.
15. Fitz-Hugh, T., Med. Clin. North America, 37, 1811, 1953.
16. Discombe, G., Practioner, 170, 373, 1953.
17. Mallory, T.B., Gall, E.A., and Brickley, W.J., J. Indust. Hygiene, 21, 355, 1939.
18. Wells, H.G., Physiol. Reviews, 1, 44, 1924.
19. Hamilton, A., Arch. Path., 11, 434, 1931.

20. Hunter, F.T., J. Indust. Hygiene, 21, 331, 1939.

21. Selling, L., Johns Hopkins Hospital Rep., 17, 81, 1916.

22. Steinberg, B., Blood, 4, 550, 1949.

23. Kracke, R.R., Amer. J. Clin. Path., 2, 11, 1932.

24. Bruno, G., Clin. Med. Ital., 65, 583, 1934.

25. Hamilton, A., Arch. Path., 11, 601, 1931 (Bibliography).

26. Fontana, G., J. Clin. Med., 2, 93, 1921.

27. Butt, E.M., Hoffman, A.M., and Soll, S.N., Arch. Int. Med., 64, 26, 1939.

28. Wooley, D.W., J. Biol. Chem., 157, 455, 1945.

29. Wooley, D.W., A Study of Antimetabolites. John Wiley and Sons, Inc., New York, 1952.

30. Heinle, R.W., Welch, A.D., George, W.L., Epstein, M., and Pritchard, J.A., Proc. Central Soc. Clin. Res., 20, 7, 1947.

31. Bergmann, E.D., Nature (Lond.), 170, 931, 1952.

32. Dameshek, W., and Colmes, A., J. Clin. Invest., 15, 85, 1936.

33. Moeschlin, S. and Wagner, K., Acta, Haematol., 8, 29, 1952.

34. Bigelow, F.S., and Desforges, J.F., Amer. J. Med. Sc., 224, 274, 1952.

35. Landsteiner, K., Specificity of Serological Reactions. Harvard University Press, Cambridge, 1945, p. 157.

36. Landsteiner, K., and Jacobs, J., J. Expt. Med., 63, 813, 1935.

37. Phillips, B., Brit. J. Dermat., 58, 213, 1946.

38. Gerber, I.E., and Gross, M., J. Immunol., 48, 103, 1944.

39. Samson, I.W., and Götz, H., Ztschr. f. d. ges. exper. Med., 52, 121, 1926.

40. Gell, P.G.H., Brit. J. Expt. Path., 27, 267, 1946.

41. Editorial, Lancet, 1, 31, 1954.

42. Sturgeon, P.J. A.M.A., 149, 918, 1952.

43. Salley, R.M., Cartwright, G.E., and Wintrobe, M.M., J.A.M.A., 149, 914, 1952.

44. Smadel, J.E., Amer. J. Med., 7, 671, 1949.

45. Morgan, I.M., J. Expt. Med., 85, 131, 1947.

46. Freund, J., Stern, E.R., and Pisani, T.M., J. Immunol., 57, 179, 1947.

47. Dubois, R.J., Bacterial and Mycotic Infections of Man. J. B. Lippincott Co., Philadelphia, 1952 (Bibliography).

48. Lipton, M.M., and Freund, J., J. Immunol., 70, 326, 1953.

49. Bjorklund, B., and Hellstrom, L., Acta Med. Scand., 139, 122, 1950.

50. Bracco, M., Curti, P.C., and Masera, N., Acta Hematol., 5 - 6, 91, 1951.

51. Benko, A., Szabo, T., Soltesz, R., and Reinhold, A., Acta Med. Scand., Fasc. 1, 3, 1953.

52. Moeschlin, S., Meyer, H., Israels, L.G., and Tarr-Gloor, E., Acta. Hemat., 11, 73, 1954.

53. Linke, A., and Ulmer, W. Quoted by Moeschlin (52).

54. Palmer, J.G., Cartwright, G.E., and Wintrobe, M.M., Amer. J. Physiol., 171, 385, 1952.

55. Morgan, P., Watson, D.W., and Cromartie, W.J., Proc. Soc. Expt. Biol. and Med., 80, 512, 1952.

56. Dews, P.B., and Code, C.F., J. Immunol., 70, 199, 1953.

57. Kabat, E.A., and Mayer, M.M., Experimental Immunochemistry. Charles C. Thomas, Springfield, 1948.

58. Bunting, C.H., Univ. Penn. Med. Bull., 16, 200, 1904.

59. Dameshek, W., and Schwartz, S., Amer. J. Med. Sc., 196, 759, 1938.

60. Forssman, J., Biol. Z., 37, 78, 1911.

61. Wilson, G.S., and Miles, A.A., Topley and Wilson's Principles of Bacteriology and Immunity. William and Wilkins, Baltimore, 1946.

62. Mushatt, C.W., Reisner, E.N., Jr., Weiner, L., Nakashima, R., and Collett, J., J. Lab. and Clin. Med., 42, 73, 1953.

63. Gruhzit, O.M., Fisken, R.A., Reutner, T.F., and Martino, E., J. Clin. Invest., 28, 943, 1949.

64. Bratton, C., Parke, Davis and Company, Personal Communication.