NORMAL VALUES AND CHROMIUM-INDUCED CHANGES IN BLOOD PHYSIOLOGY OF RAINBOW TROUT, SALMO GAIRDNERII

By

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ABSTRACT

This study presents a physiological approach to the toxicity problems encountered in stream pollution control. Although this work deals with the effects of potassium chromate on rainbow trout, the ideas presented might be projected into the general study of chronic toxicity of waste products to fish.

The normal hatchery-raised rainbow trout was examined in order to establish the following control values: (a) hematocrit, 31.8 ml./100 ml.; (b) red blood cell count, 1.11 million cell/cmm.; (c) hemoglobin, 6.51 g./100 ml.; (d) red blood cell length, 14.69 μ.; (e) plasma volume 2.13 ml./100 g.; (f) blood volume, 3.25 ml./100 g.; (g) kidney weight, 0.77 g./100 g. of body weight; (h) liver weight, 1.02 g./100 g. of body weight; (i) spleen weight, 0.24 g./100 g. of body weight; and (j) total body water, 77.4 g./100 g. In addition to the above, the kidney, liver and spleen weights; total body water; and plasma and blood volumes were found to correlate significantly with body weight. The regression equations are given.

In order to obtain the above results, mammalian methods had to be adapted to small fish which ranged between 10.0 cm. to 17.0 cm. in length with a mean weight of 19.0 grams. As a result of

this, an extensive and detailed section titled "Materials and Methods" is included.

In order to fix a working level of potassium chromate, a 24-hour median tolerance limit of 100 mg. Cr./l. was determined, and all experiments were done at a potassium chromate concentration equivalent to 20 mg. Cr./l.

Tracer experiments implicated the spleen and bile as concentrating chromium above the level of the nonspecific tissue of the caudal peduncle. As a result, it was theorized that the reticuloendothelial system of the spleen and the liver were instrumental in the elimination of chromium from the body by way of the bile.

There was no evidence of a change in weight of the kidney, liver, or spleen, or a change in total body water due to exposing the fish to 20 mg. Cr./l. for 24 hours.

Further investigations showed that the hematocrit rose from a control value of 31.8 to 43.8 in chromium-exposed fish in 24 hours. With the use of erythrocyte counts and hematocrits, it was calculated that approximately 59 percent of the rise in hematocrit was due to increase in cell volume, and 41 percent was due to increase in cell number. It was shown that the spleen did not contribute significantly to this increase in cell number.

When the hematocrit rises, there must be an increased blood volume and/or decrease in plasma volume. Neither of these events could be shown to take place under the experimental conditions.

Due to the fact that potassium chromate contains a considerable amount of potassium ions, the influence of this ion was investigated. It was assumed on the basis of the experimental data that the potassium ion, in addition to the chromium, is a potential influence upon the results obtained at potassium chromate concentrations equivalent to 20 mg. Cr./l.

Assuming the rise in hematocrit was an index of chronic toxicity, it was determined that the hematocrit rose to its greatest degree at concentrations equivalent to between 2 mg. to 4 mg. Cr./l. This fact was of interest because, although the 24-hour Tlm, a standard index of toxicity, was 100 mg. Cr./l.; the above data indicated physiological changes take place at a far lower concentration.

With the use of splenectomized fish, it was found that the spleen has a tendency to act, in a limited capacity, as a buffer mechanism against sudden hemoconcentration. This was evidenced by a significant rise in hematocrit of splenectomized fish in tap water when exposed to ten minutes of operative and anesthetic stress.

ACKNOWLEDGMENTS

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INTRODUCTION

The problem of stream and lake pollution control is a diverse one touching upon many facets of the biological and physical sciences. The prevailing discipline is aimed at the detection of pollution already in the waters with control coming after legal proceedings. As water resources become utilized to their fullest extent by municipalities and industries as well as for their recreational and esthetic values, the attitude is changing rapidly to one of control before pollution occurs. This aspect of control brings forth many new questions such as, "How much of a waste can be discharged into a particular stream before it will adversely affect the aquatic life?" This is a question industry is asking the regulatory agency more and more; the answer to which, if slightly wrong, can cause either loss in value of a stream or a very real monetary loss to the industry.

Contemplation upon this question will reveal three interacting variables. The waste, the stream, and the form of aquatic life, each of which must be given consideration. First the waste: Ignoring the purely chemical dilemma which arises from an attempt to determine its ionic and molecular constituents and focusing

attention on its biological implications, we find that variation in terms of concentration is further complicated by the antagonistic and synergistic action of various metallic cations. It has long been known that the toxicity of some metal cations to aquatic organisms is counteracted, or antagonized, by other metal cations in mixed salt solutions (Heilbrunn, 1938). An excellent example of this is Ringer's solution in which the sodium, calcium, and potassium ions are balanced in such proportions that the specific toxicity of each is neutralized.

Loeb and Wasteneys (1911, 1915) reported some of the early studies of antagonism of the principle cations of sea water relating to fish. Garrey (1916) followed immediately with his work on the average longevity of minnows of the genus Notropis in various mixtures of sodium, potassium, calcium, and magnesium chlorides in distilled water. He found that minnows in 9,000 mg. NaCl/l. would live two days, but the addition of the sodium chloride to 0.001 M. calcium chloride or more, prolonged the period to eight to ten days. In turn, he found the calcium ion to be antagonistic to potassium and magnesium. A high concentration of sodium chloride counteracted the toxicity of calcium chloride and potassium chloride, and magnesium chloride was antagonistic to potassium chloride.

Other workers (Powers, 1921; Krüger, 1928; Gueylard, 1923, 1924; Ramult, 1928a 1928b; Young, 1938) investigated the toxicity relationships of these ions upon sticklebacks, minnows, and other species including salmon eggs and found the same over-all results.

In addition, Ellis (1937) showed that sodium nitrate decreased the toxicity of cupric sulfate and the combination of sodium nitrate and calcium chloride was even more antagonistic. Much work has been done on antagonism (Ellis and Ladner, 1935; Jones, 1938), but in reality it is not greatly concerned with the problem at hand and thus, it is considered only briefly here.

Synergism between various cations likewise has been shown to exist. Bandt (1946) demonstrated that mixtures of the sulfates of nickel and zinc, copper and zinc, and of copper and cadmium were up to five times more toxic than they would have been if the toxic effect of the metals were simply additive. Doudoroff (1952) substantiated the evidence on the synergistic action between copper and zinc.

We can project the ideas brought out in this brief discussion of the interactions of the component parts of the waste and see that the stream water itself is a variable in any consideration of toxicity levels. Calcium, for example, is an important ion in naturally occurring waters; its presence in large and small degrees depends

upon the geological formation of the watershed. It contributes largely to the hardness of the water, which in turn is directly related to the alkalinity, each being important to the potency of a waste. In addition, the pH, other ion species, suspended solids, dissolved gases, and, if the stream is extensively utilized by industry, waste products discharged upstream contribute to the complexity of disposal problems.

Not the least to be considered in toxicity problems is the amazingly complex biodynamic cycle occurring within the water itself. Its interrelationships cause potomologists to compare a stream to a living organism. Water, a most important sustaining factor of life, holds confined within its natural basins many floristic and faunistic phyla. The sportsman, an influential factor in stream control, thinks in terms of fish production, while the aquatic biologist must consider the vast interdigitating food chain which ecologists term the "food web." Even though fish may live through some catastrophic changes in their environment, the delicately balanced relationships between trophic levels may be upset sufficiently to make the habitat unsuitable for them and other forms long after the disaster has passed. Not only is the problem viewed from a negative attitude in which important species disappear from one or more trophic levels, but it can be a positive situation.

The degradation of the stream may become a favorable factor for a particular species which in turn increases its numbers considerably out of proportion forming a caricature of the original biodynamic picture.

Having briefly discussed the problem of stream pollution in general, we shall consider the present approach to the question: "How much of a waste can be discharged into a particular stream before it will adversely affect the aquatic life?" This question is being answered, from an industrial waste aspect, by the toxicity bioassay. Doudoroff et al. (1951), commissioned by the Federation of Sewage and Industrial Wastes Associations, have attempted to standardize the performance of these tests; however, the literature at present is filled with as many methods as results.

The most obvious faults in the bioassay methods are (1) they are carried out under critical concentrations of the wastes without consideration of chronic levels; (2) the majority of the work is done on fish, and the effect of the wastes on lower forms is ignored; and (3) they display a complete lack of concern for site of entry and the mode of action of the toxic ions within the fish.

Due to the variables discussed, the literature shows a wide variation in the tolerance of fish to chromium. A summary of this literature is in Table 1. In order not to confuse this discussion

TABLE 1

A BRIEF SUMMARY OF THE TOXICITY LITERATURE

Animal	Compound	Results	Reference
Bluegill Sunfish	Na_2CrO_4	24-hr. TLm--930 mg. Na_2CrO_4/l.	Abegg, 1950
	$Na_2Cr_2O_7$	24-hr. TLm--728 mg. $Na_2Cr_2O_7$/l.	
Stickle-backs	$Cr_2(SO_4)_3$	Lethal conc. limit--1.2 mg. Cr./l.	Jones, 1939
Rainbow Trout	K_2CrO_4	Toxicity [100 x (1/time)] --0.050±0.66 $min.^{-1}$; 2000 min. test. at 50 mg. Cr/l.	Grindley, 1946
	K_2CrO_4	Toxicity--0.051±0.044 $min.^{-1}$; 1946 min. text. at 50 mg. Cr/l.	
Young Eels	$KCr_2(SO_4)_2$	Survival time at 5.2 mg. Cr/l. averaged 18.7 hours	Oshima, 1931
	K_2CrO_4	Survival time at 520 mg. Cr/l. averaged 12.4 hours	
	$K_2Cr_2O_7$	Survival time at 520 mg. Cr/l. averaged 5.35 hours	
Minnows	Trivalent Cr as sulfate	40 mg. Cr/l. is a minimum fatal concentration in 6 hours	LeClerc and Devlaminck, 1950
Trout	$K_2Cr_2O_7$	6 hours' exposure to 100 mg. $K_2Cr_2O_7$/l. fatal within 12 hours after removal	Rushton, 1921
Large-mouth Bass	K_2CrO_4	48 hr. TLm; 195 mg. Cr/l.	Fromm and Schiffman, 1958

TABLE 1 (Continued)

Animal	Compound	Results	Reference
Daphnia	K_2CrO_4	50% immobilized in 16 hours; < 0.6 mg./l.	Anderson, 1944
	Na_2CrO_4	50% immobilized in 48 hours; < 0.32 mg./l.	Anderson, 1946
	$Na_2Cr_2O_7$	50% immobilized in 48 hours; << 0.31 mg./l.	
Flora	Trivalent chromium	Toxic at 6 mg./l. or less	Rudolfs et. al., 1950

with a detailed description of these works, the reader will find this review of literature in Appendix I.

A comparison of the data found in the literature is somewhat hazardous, since they are reported in many ways from median tolerance limits to toxicity per minute and done under a variety of conditions. It is obvious that the variants, the animals, the receiving water, and the molecular form of the waste all contribute to the confusion, with the inevitable result that the bioassay data are of practical use only to the originator of the experiment, and then only in a given situation.

In an attempt to remedy this, it was felt that one must study the individuals within the population undergoing the stress of a toxic

environment. This approach, of necessity, led directly to the field of physiology.

The literature dealing with changes in the physiology of fish under chromium stress is almost completely lacking. Abegg (1950), under conditions described in Appendix I, studied changes in tissue fluids and blood specific gravity of bluegills brought on by exposure to solutions of sodium chromate and sodium dichromate. By removing a strip of dorsal muscle tissue, weighing it, desiccating it in an oven at a temperature of 110° C. for 24 hours and reweighing the dry tissue, he determined the tissue fluid volume. This was expressed as percentage of water of the wet-tissue weight.

Blood specific gravity was determined by use of the falling-drop densiometer. This method combines the merits of small sample size, 0.01 cmm., with speed and accuracy.

Blood for the determination was obtained from a pithed fish's bulbus arteriosus. By inserting a 3/4-inch needle tube broken from a 23-gauge hypodermic needle in the bulbus, it was found that the action of the heart pumped the blood out the tube so that it could be collected on a paraffin block. The data are reported in Table 2.

Abegg felt that the various physiological reactions of the fish to the salt solution was difficult to explain unless one assumed

TABLE 2

PHYSIOLOGICAL REACTIONS OF THE FISH TO THE SALT SOLUTION

Chemical	No. of Fish	Hours Exposed	$\bar{X}$ % Tissue Fluid	Standard Deviation	P Value[a]	Pct. Change[b]
			Tissue Fluids			
Na_2CrO_4	10 Control	24	81.22	0.8267		
	10 Test	24	81.63	0.6610	N.S.	N.S.
$Na_2Cr_2O_7$	10 Control	13	80.30	0.7457		
	11 Test	13	82.24	1.4419	0.01	+2.42
			Blood Specific Gravity			
Na_2CrO_4	10 Control	24	1.0441	0.001451		
	10 Test	24	1.0452	0.006406	N.S.	N.S.
$Na_2Cr_2O_7$	10 Control	13	1.0399	0.011361		
	9 Test	13	1.0372	0.003501	0.05	-0.375

[a]Probability based on Fisher's t-test values. N.S. denotes no significant change.

[b]Calculated on the basis of the control values equaling 100 %.

that the ions enter the fish's body. This is possible through several pathways: the skin, gills and oral membranes, and by ingestion.

Abegg (1949) made a study of the effect of salt solutions on the mucous covering of bluegills. The salt concentrations used were the same as those reported in his 1950 paper. Phenol red was

added to the test solutions to a concentration of 1:10,000. The fish were removed after one hour's exposure and rinsed off under running tap water for three minutes. The body surfaces of all experimentally treated fish were stained with the dye. The untreated controls remained unchanged. He felt this showed an alteration of the mucous cover which might then have permitted the ions to penetrate the skin. Abegg found that both sodium carbonate and sodium dichromate caused an increase in tissue fluid content of the musculature, and in both cases they caused the precipitation of the mucous covering of the body.

In the same paper, using H. W. Smith's phenol red technique, Abegg demonstrated that the fresh-water stenohaline teleost drinks water under normal conditions as well as in solutions of higher osmotic pressures.

In addition to the conclusions that sodium dichromate caused an increase in tissue fluids, Abegg stated the size of fish used in a toxicity study is an important factor in the interpretation of the data; for he found that the larger fish (20 gm. to 35 gm.) were more tolerant than the small fish (4 gm. to 10 gm.).

Fromm and Schiffman (1958), in their work with largemouth bass, concluded that exposure to 94 mg. Cr/l. caused initially a slight, but not highly significant increase, in the oxygen consumption

of the animal. This was followed by a gradual decline to 27 percent below normal after 68 hours' exposure.

Also, they found, by microscopic examination of the intestine, severe pathological changes immediately posterior to the pyloric caeca, which in all probability completely destroyed its digestive functions.

In addition, coagulated strings of mucus from the anus of all experimental fish were observed prior to death. It was theorized that chromium entered the gills and was excreted, in part, by the liver via the bile. This chromium-laden bile caused the intestinal damage.

It is interesting to note that no damage to the gill tissue was reported, although the common criterion is that the heavy metals kill by precipitation of the mucus covering of the gills. They did note a coughing reflex in the fish before death, but the exact significance of this is not known.

Bearing in mind the literature as reviewed herein, the individual interested in the effects of chromium wastes on fish, or other aquatic organisms, can obtain little help with the problems at hand, since the work done applied to a particular set of conditions and not to generalities. To reiterate, it was felt that a physiological-pharmacological approach to the problems of stream pollution would

produce a better understanding of the many influencing factors. For example, if we understood how chromium enters the fish, and if we knew the distribution and the mode of action of the toxic ion once it is within the organism, we could better understand the limits of toxic stress.

This project was approached with these ideas in mind, not as an answer to toxicity problems as a whole, but as one piece in a complex puzzle. Like the conventional bioassay, this experiment fixes the variables; one water, one waste, and one species of animal is used. Unlike the bioassay, it is designed to promote an understanding of the stress the individual fish is experiencing. Another difference--perhaps the most important--is the fact that this experiment deals not with critical concentrations, but sublethal doses of chromium.

To orient this work with the literature and to use as a baseline for the project as a whole, a 24-hour median tolerance limit was determined. The subsequent experiments were all performed at one-fifth of this limit. Once the working concentration level was determined, pilot experiments using the radioisotope Cr^{51} implicated the circulatory system and the liver as a path of the chromium within the trout. It is safe to assume that the blood is instrumental in the transport of the toxic agent from its entrance into the animal

until its final exit. Therefore, it would be the first tissue that might exhibit changes due to the stress. Under this assumption, this thesis is predominantly directed at chromium-induced changes in blood, and includes determinations of hematocrits, hemoglobins, cell counts, plasma volumes, and blood volumes, among others.

MATERIALS AND METHODS

The rainbow trout used throughout this work were obtained from the Michigan Department of Conservation through the cooperation of Messrs. Edward Bacon and Ralph Marks. The fish were transported in plastic bags in lots of one hundred from the Wolf Lake Hatchery to the laboratory. Fifty trout were placed in a small amount of water in each bag, after which the bag was inflated with oxygen and sealed. Ice was placed around the outside of the bags, when the temperatures warranted it, to keep the fish at 15° C.

In the laboratory the fish were held in four 26-gallon glass aquaria kept in a constant temperature room (Figure 1). This room was illuminated continually by fluorescent lights overhead and on each holding tank. Constant illumination was utilized because each time the lights were turned on over the holding tanks the fish would become excessively excitable, thus introducing a variable that might affect the final results.

The water used throughout the experiments was held at 14° C. to 15° C. in the holding tanks and in the experimental tanks, and was tap water which had a hardness of 334 mg. of $CaCO_3$/l., a total alkalinity of 204 mg. of $CaCO_3$/l. and a pH of 8.5 to 8.8.

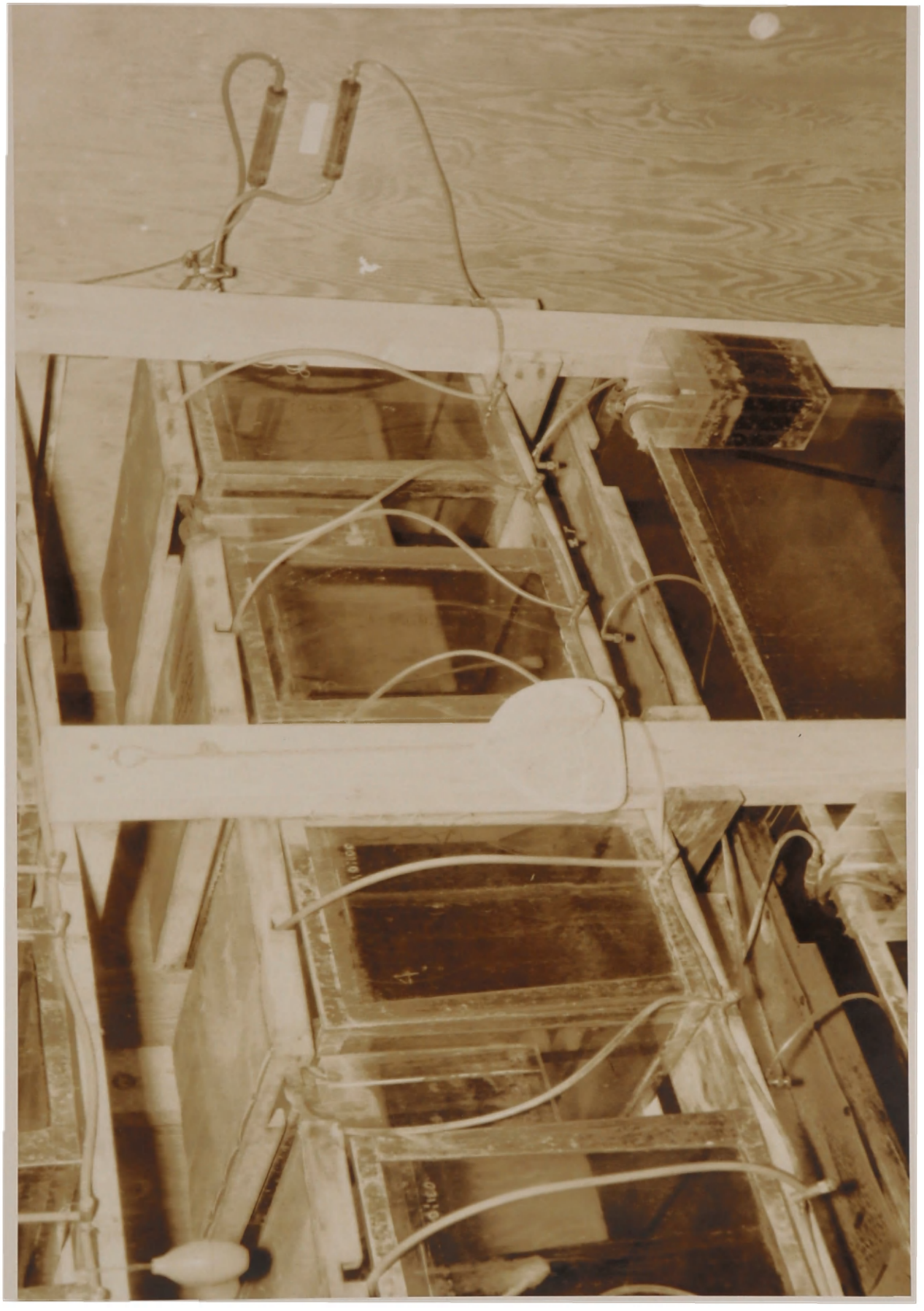

Figure 1. Experimental aquaria in constant temperature room.

On each Monday, Wednesday, and Friday the fish were fed dried trout pellets developed by the Michigan Department of Conservation for use in their hatcheries.

Chemical Methods

Total residue (Standard Methods, 1955)

Total residue, or total solids, was done by evaporation. Fifty milliliters of sample were placed in a weighed dish and evaporated to dryness at temperatures between 95° C. and 105° C. The dish was reweighed and the difference calculated. The total residue in mg./l. was found by the following equation:

mg. total residue/l. = (difference x 1000)/(ml. of sample)

Alkalinity (Standard Methods, 1955)

Alkalinity was done by titration. Fifty milliliters of sample were titrated to pH 8.3, using phelolphthalein indicator, with 0.02 N. hydrochloric acid, after which it was further titrated using Fleisher Methyl Purple Indicator to a purple endpoint at a pH of 4.8.

The phenolphthalein alkalinity was calculated as mg. of $CaCO_3$/l. by the following formula:

mg. $CaCO_3$/l. = (ml. standard acid x 1000)/(ml. of sample)

The total alkalinity was also expressed in mg. of $CaCO_3$/l. and was calculated in the same manner using the total milliliters of standard to bring the solution to a pH of 4.8.

The total alkalinity of a water is made up of three types; namely, the hydroxide, carbonate, and bicarbonate alkalinity found in the approximate relationships as illustrated in Table 3, taken from Standard Methods (1955).

De Martini's (1938) equations for the determination of the three types of alkalinities and H_2CO_3 are as follows:

$$(1)\quad (HCO_3^-) = \frac{[Alk. + (H^+) - \frac{K_w}{(H^+)}]}{1 + 2\frac{K_2}{(H^+)}}$$

$$(2)\quad (CO_3^-) = \frac{K_2}{(H^+)} \times \frac{[Alk. + (H^+) - \frac{K_w}{(H^+)}]}{1 + 2\frac{K_2}{(H^+)}}$$

$$(3)\quad (H_2CO_3) = \frac{(H^+)}{K_1} \times \frac{[Alk. + (H^+) - \frac{K_w}{(H^+)}]}{1 + 2\frac{K_2}{(H^+)}}$$

$$(4)\quad (OH^-) = \frac{K_w}{(H^+)}$$

"Alk." alkalinity is in equivalents of titratable base per liter. K_1 and K_2 are the first and second ionization constants of carbonic acid and K_w of water.

TABLE 3

THE RELATIONSHIP OF THE THREE TYPES OF ALKALINITY TO THE TOTAL AND PHENOLPHTHALEIN ALKALINITY

Results of Titration	Hydroxide Alk. as $CaCO_3$	Carbonate Alk. as $CaCO_3$	Bicarbonate Alk. as $CaCO_3$
P = 0	0	0	T
P = 1/2 T	0	2P	T-2P
P = 1/2 T	0	2P	0
P = 1/2 T	2(P-T)	2(T-P)	0
P = T	T	0	0

T = total alkalinity; P = phenolphthalein alkalinity.

Moore (1939) transformed these equations into graphic form. These were used in this work.

Hardness (Schwarzenbach Titration)

Hardness determinations were made by titration using reagents manufactured by the Hall Laboratories, Inc., and purchased from Calgon, Inc., Chicago, Illinois. This is a versenate titration based upon the methods invented by Dr. G. Schwarzenbach and associates in Switzerland. Samples of 50 ml., with 1 ml. of Hardness Buffer Solution added, were titrated with Hagan Hardness

Titration Solution (1 ml. = 20 mg. $CaCO_3$/l.) using the hardness indicator which changes from red to blue at the endpoint. Hardness was calculated as follows:

Total hard. as mg. $CaCO_3$/l. = 20 x burette reading in ml.

Chromium (Saltzman, 1952)

Samples from the aquaria were diluted, when necessary, so that the amount analyzed, 1 to 10 ml., would contain from 5 to 15 micrograms of chromium. This was ashed in a 125-ml. Phillips beaker by adding 0.5 ml. glass distilled reagent grade concentrated nitric acid, and 0.25 ml. of 40 percent sodium bisulfate, then evaporated to dryness on a Lindberg type hot plate. At this point Saltzman recommends repeating the addition of acid and ashing again, but it was found that this was unnecessary under the conditions existing during these tests. Ten milliliters of 0.5 N. sulfuric acid, made up with double distilled water, were added and swirled to dissolve the ash. When the acid was added to the hot ash it went into solution with much less difficulty than did the cooled ash.

Any water, including reagents made up with water, added to the samples at this point or afterwards, must be double glass distilled. This prevents reduction of the hexavalent chromium which is formed by the ashing and subsequent steps. Only the hexavalent

chromium will develop the color of the s-diphenylcarbazide reagent.

To the acid solution of the ash is added 0.5 ml. of 0.1 N. potassium permanganate; it is then covered and heated on the hot plate at approximately 100° C. for 20 minutes. Saltzman's method calls for the addition of more permanganate if the pink color disappears, but once again this step was found unnecessary. At the end of this time the permanganate is decolorized by the addition of 5 percent sodium azide at the rate of 1 drop every 10 seconds, swirling after each drop. Three to 5 drops were usually enough to destroy any brownish tint, and any excess was avoided since this would reduce the chromium. With the samples once again colorless, they were removed immediately from the heat and placed in a tray of cold water. The samples now may be filtered, if necessary, or transferred directly into 25 ml. volumetric flasks using double distilled water as a wash liquid. Once the samples are in the volumetric flasks, color development can proceed.

Color was developed by adding 1.0 ml. of s-diphenylcarbazide reagent. This reagent was made up by dissolving 10 grams of phtalic anhydride in 175 ml. of redistilled 95 percent ethyl alcohol, warming to effect solution. To this is added 0.625 grams s-dyphenylcarbazide dissolved in 50 ml. of redistilled alcohol. Finally,

this combination is made up to 250 ml. with alcohol. This reagent is quite stable and was stored in a brown bottle under refrigeration for 8 months. Only occasional restandardization was necessary.

One minute after the developer was added, 2.5 ml. of 4 M. sodium dihydrogen phosphate was added as a buffer and the sample made up to 25 ml. If iron was present, the buffer could be added before the s-diphenylcarbazide and allowed to stand 15 minutes before reading. This would eliminate interference due to small amounts of iron.

The pink color was read at a wavelength of 540 mμ. within 30 minutes on a Bausch and Lomb Spectronic 20 colorimeter.

A potassium chromate solution was used to standardize the determination and was made by dissolving 0.3734 grams of potassium chromate in 1 liter of redistilled water. This contained 100 μg. of chromium per milliliter. Standard curves of micrograms hexavalent chromium versus percent transmission were made up approximately every two weeks when many analyses were being made, but if chromium determinations were done periodically, a curve was made with each series of determinations.

Hexavalent chromium was determined by procuring a properly diluted sample in a 25 ml. volumetric flask and adding the s-diphenylcarbazide reagent and buffer as above. The samples were

then diluted to 25 ml. with double distilled water and read colorimetrically using the same standards as above.

The accuracy of the test was determined by making up 2 μg. Cr^{+6}/ml. standards using both potassium chromate and potassium dichromate. Three 10 μg. samples from each solution were analyzed for total chromium and hexavalent chromium, each sample being read in the colorimeter three times. The results of these analyses appear in Table 4. The means and standard deviations of these tests are shown in Table 5.

Due to the fact that the samples agree well within themselves, it was felt that the deviation from the standard of 10 μg. was due to the dilution and measurement methods and not the analytical procedure. Taking 10 μg. as the mean of the population ($\bar{\mu}$) and using the series of samples with the greatest variation (s^2 = 0.206, $\bar{X}$ = 10.9) it was found that the standard error of this group is $\bar{\mu} \pm 0.37$. Making the assumption, which is not substantiated by experimental data, that this error would not change appreciately with greater dilutions, it was felt that this accuracy was sufficient for testing all water samples.

It was also found by using $\bar{\mu}$ = 10 and the results of the potassium chromate samples that a Fisher "t" test showed no evidence of a difference between total and hexavalent chromium

TABLE 4

RESULTS OF TESTS TO DETERMINE THE ACCURACY OF THE CHROMIUM METHOD

Reading on Colorimeter	Total Chromium			Hexavalent Chromium		
	Sample Number			Sample Number		
	1	2	3	1	2	3
	$K_2Cr_2O_7$					
1	11.6	10.8	10.6	10.6	11.0	11.0
2	11.5	10.8	10.6	10.6	10.8	11.0
3	11.5	10.8	10.4	10.6	10.6	11.0
	K_2CrO_7					
1	10.2	10.8	10.6	10.0	10.6	10.2
2	10.6	11.8	10.6	10.2	10.6	10.2
3	10.4	10.6	10.8	10.2	10.8	10.0

TABLE 5

MEANS AND STANDARD DEVIATIONS OF TESTS TO DETERMINE THE ACCURACY OF THE CHROMIUM METHOD

Chromium	$K_2Cr_2O_7$		K_2CrO_7	
	$\bar{X}$	s	$\bar{X}$	s
Total chromium	10.9	0.454	10.6	0.235
Hexavalent chromium	10.8	0.200	10.3	0.285

methods. Upon this basis, it was assumed that the chromium added to the aquaria remained for the most part in the hexavalent form, and later experiments upheld this theory.

Radioactive Isotope Methods

The radioisotope Chromium 51 was used in the tracer experiments. This element was supplied by the National Laboratory, Oak Ridge, Tennessee, in the form of $CrCl_3$ in HCl solution with a concentration of 22.85 $\pm$ 10 percent mg./ml. and a specific activity of 1760 mc/g. This, the trivalent form, had to be oxidized to the hexavalent form for use in this problem. This transformation was carried out in the following manner. A sample of approximately 50 µg. of Cr^{51} was made basic with 2 ml. of 6 N. Sodium hydroxide and 1 ml. of 3 percent hydrogen peroxide was added. This was heated at 110° C. for 5 minutes, after which it was boiled for one hour to drive off the excess peroxide. At the end of the boiling period, while the solution was still warm, 0.2 ml. more of the NaOH was added and swirled. If bubbles were given off, the solution was boiled again until no bubbling was evident. When no bubbling occurred, the solution was neutralized with 2 ml. of 6 N. hydrochloric acid, cooled, and used.

The test animals were always exposed to the radioactive isotope by making a dilution of their environmental water. Water samples of 1 ml. were analyzed for activity by evaporating them in porcelain planchets with a diameter of 35 mm. Gamma ray counts of these preparations were made with a Nuclear Instrument and Chemical Corporation Model 183 Scaler equipped with a Tracerlab tube of the scintillation type.

Organ counts were made by weighing the fresh tissue on a Roller-Smith balance and dry ashing them on the porcelain planchets in a muffle furnace at 500° C. When the residue turned white or light gray, the planchets were removed from the oven and cooled. Concentrated nitric acid was added to dissolve and/or suspend the ash, the resultant solution being evaporated slowly on a hot plate. This left a thin film of the ash evenly distributed on the planchet, which was counted as above.

Both water and organ counts were made either as counts per five minutes, or as minutes per 400 counts, corrected for decay and reported as counts per minute.

Biological Methods

Fish bioassays (Doudoroff et al., 1951)

This method set up by the Committee on Research, Subcommittee of Toxicity, Section III of the Federation of Sewage and

Industrial Wastes Associations, was followed as closely as practicable; deviations were made only when the researcher felt them to be of no effect on the final results.

Since this is a standard procedure in the field ot toxic waste control, but unknown in pure physiological work, it is felt that a brief outline of the method is justifiable in this work.

Fish. Fish being used in bioassay tests should be native to the watershed receiving the wastes, and should be neither a tolerant nor a highly intolerant species, but rather, one with a record of median tolerance. The following families are recommended:

Centrarchidae (sunfishes, basses, crappies)

Salmonidae (trout, charrs, salmons)

Cyprinidae (true minnows) exclusive of carp and goldfish

Catostomidae (suckers)

Test animals should be obtained from any single source and brought to the laboratory at about the same time. The length of the largest fish should not be more than 1.5 times the length of the smallest. Doudoroff recommended fish averaging less than three inches, but this was not adhered to because of the difficulty that would be encountered in performing other physiological tests. Fish used throughout the work averaged 13.6 cm. in length with a range of from 10.0 to 17.0 cm. and a mean weight of 19.0 grams.

The test animals should be acclimatized to laboratory conditions for at least one week, preferably ten days or longer. During this time they are to be fed at regular intervals at least three times a week. Fish should not be fed two days before, nor during the test.

The percentage of fish dying or seriously diseased during the acclimatization period should not exceed 10 percent.

The experimental water (diluent). Water from the stream receiving the waste or artificial stream water of similar quality with respect to the calcium, magnesium, sulfate, total dissolved solids, and total alkalinity is recommended. Since the research project reported herein is not a problem specific to a particular stream, tap water was decided upon as the diluent.

Other experimental conditions. The test should be performed at uniform temperatures between 68° F. and 77° F. (20° C.-25° C.) for warm water fish and 54° F. to 64° F. (12° C.-18° C.) when cold water fish are used. The range should not exceed 2° F. or 1° C. above or below the chosen temperature.

The test container or aquaria should be of glass and of such a size that depth of water is never less than 6 inches. The dissolved oxygen content of the water should never be less than 4 ppm

for warm water fish or 5 ppm for cold water fish. Aeration, under some conditions, is not recommended due to the fact that this may drive out gaseous or volatile components of a waste.

At least ten animals should be tested for each dilution; the total weight of all fish in a test container should not exceed 2 grams per liter, and 1 gram per liter or less being preferred.

Aeration and filtration were both used in these experiments after due examination of their effect on the dissolved chromium. Aeration was accomplished by compressed air introduced into the tanks via a stone airbreaker. Aquarium filters of glass wool and gravel were used to clear the water of suspended solids. Before using these apparatus, which were contrary to recommended procedure, three aquaria were set up as follows:

Aquarium 1 No filter, no aeration

Aquarium 2 No filter, aeration

Aquarium 3 Filtration, aeration

The water in each of these tanks contained 10 mg. Cr^{+6}/l. The total and hexalent chromium, and total alkalinity measured at the start of the experiment and 24 hours, 48 hours, and 5 days after the addition of chromium. The results of these tests are shown in Tables 6 and 7.

TABLE 6

RESULTS OF TESTS TO DETERMINE WHETHER FILTRATION OR AERATION HAD AN EFFECT ON THE CHROMIUM CONCENTRATION

Tank No.	Total Chromium			Hexavalent Chromium			pH
	Sample A (mg./l.)	Sample B (mg./l.)	$\bar{X}$	Sample A (mg./l.)	Sample B (mg./l.)	$\bar{X}$	
			Start				
1	9.6	9.8	9.7	9.6	---	9.6	8.03
2	9.4	10.0	9.7	9.2	---	9.2	8.34
3	9.8	10.0	9.9	9.8	---	9.8	8.58
			24 Hours				
1	10.6	10.2	10.4	10.4	10.6	10.5	8.34
2	10.6	10.7	10.6	10.7	10.7	10.7	8.83
3	10.4	10.2	10.3	10.7	10.6	10.6	8.80
			48 Hours				
1	10.2	10.2	10.2	10.6	10.6	10.6	8.33
2	10.0	10.0	10.0	10.4	10.6	10.5	8.83
3	10.0	10.0	10.0	10.2	10.1	10.2	8.85
			5 Days				
1	10.6	10.6	10.6	---	---	---	8.38
2	10.4	10.2	10.3	---	---	---	8.91
3	10.1	10.6	10.3	---	---	---	8.90

TABLE 7

RESULTS OF TESTS TO DETERMINE WHETHER FILTRATION OR AERATION HAD AN EFFECT ON ALKALINITY

Tank Number	Total Alkalinity		
	Sample A	Sample B	Mean
	Start		
1	220	224	222
2	220	220	220
3	212	212	212
	24 Hours		
1	224	224	224
2	218	220	219
3	222	224	223
	48 Hours		
1	224	---	224
2	216	---	216
3	222	---	222
	5 Days		
1	224	---	224
2	224	---	224
3	224	---	224

It can be concluded readily from these data, even without benefit of statistical analysis, that neither filtration nor aeration altered the chromium content or total alkalinity of the water. As a result it was felt that ten fish could be kept in each 40-liter aquarium without altering the final results of the bioassay, even though this greatly exceeded the recommended number. The dissolved oxygen content was always above 5 ppm and averaged 8 ppm.

Experimental procedure. Concentrations to be tested were made up by adding potassium chromate crystals (Mallinckrodt Analytical Reagent) directly to the filled aquaria; the resultant solution was then analyzed for hexavalent chromium. Experimental concentrations selected were based on progressive bisection of intervals on a logarithmic scale. Pilot tests with few fish and a wide range of concentrations were done to establish the approximate tolerance limits before the bioassay began. A control tank of ten fish under the experimental conditions was tested simultaneously with the bioassay. If the control tank had a mortality of higher than 10 percent, the test results were considered unreliable. The fish were handled with small dipnets and measured and weighed previous to the start of the test. The tests were run for both 24 and 48 hours.

The data are reported as a 24-hour or 48-hour median tolerance limit (TLm). This is estimated by straight-line graphical

interpolation using percent survival on the ordinate and log. dose (expressed in mg. Cr^{+6}/l.) on the abscissa. In order to do this, it is necessary to have two test concentrations, one in which the surviving fraction is above 0.50 but below 0.90, and the other with a surviving fraction below 0.50 but above 0.10. It can readily be seen that the closer these fractions are to 0.50 the more accurate the estimate will be. By extending a straight line between these two points, the TLm is read in mg. Cr^{+6}/l. at the point the line crosses the 50 percent survival axis.

For the biologist interested in stating a presumably harmless concentration (C) of a waste, Hart, Doudoroff, and Greenback (1945) suggested the following equation with reservations:

$$C = (48 \text{ hr. TLm} \times 0.3)/(S^2)$$

$$S = (24 \text{ hr. TLm})/(48 \text{ hr. TLm})$$

Drawing of blood

It was found early in this work that the procuring of a valid sample of blood from small fish would prove to be a difficult procedure. The cutting off of the tail at the caudal peduncle and collecting the blood as it flowed from the caudal vein is a popular method in fish research. It was tried in the earlier pilot experiments. The high variance of the samples obtained by this method

indicated the blood was being contaminated, probably by spinal and interstitial fluids. After practice, blood was obtained by heart puncture from fish anesthesized with 300 mg. MS-222/l. (Tricaine methanesulfonate). It was virtually impossible to make a successful heart puncture through the skin in these small fish; thus, a ventral incision was made to expose the heart, and the pericardium was stripped off to free the ventricle. Next a 0.25 milliliter syringe, rinsed in 1000 USP/ml. heparin solution, and fitted with a 27 gauge needle, was inserted straight down into the ventricle at its base near the junction of the truncus arteriosus. By keeping a slightly negative pressure within the syringe each heart beat would pump blood into it. No blood sample of less than 0.1 ml. was used in these determinations, since it was felt that the residue of heparin, although as much as possible had been expelled before taking the sample, would cause a serious dilution error. The average sample was between 0.1 and 0.2 milliliter.

Hematocrits

The blood drawn by heart puncture was expressed out of the syringe onto a wax surface taking care to avoid air bubbles which might get into the blood pipettes. A van Allen hematocrit tube was filled up to the 100 mark from the pool of blood, after which it was

diluted with a 0.6 percent sodium chloride until the bulb of the hematocrit tube was three-fourths full. The tube was sealed with a spring clip and centrifuged at 2500 RPM for twenty minutes. The accuracy of this test was determined by taking four different hematocrits from a single blood sample from one fish. The resultant standard error was 0.72 ml./100 ml.

Hemoglobins

Hemoglobin content of the blood was determined as acid hematin as outlined in the Bausch and Lomb Spectron 20 Clinical Technique Manual. Twenty cubic millimeters were added to 5 milliliters of 1 percent HCl and allowed to stand one hour. Since fish blood is nucleated, the resultant solution was slightly turbic, which interfered with the colorimetric method. The nuclei were removed by centrifuging at 2500 RPM for 10 minutes, and the supernatant was read on the colorimeter at 525 mμ wavelength. The standards for human blood provided with the machine were used, and the results were reported in grams of hemoglobin per 100 grams of blood.

Like the hematocrit determination, the accuracy of this method was established by taking six hemoglobin samples from a single blood sample. The standard error was 0.32 grams hemoglobin/100 grams blood.

Red blood cell count

A standard red blood pipette was filled from the puddled blood, and a 1:200 dilution was made using 0.6 percent sodium chloride as the dilutant. A red blood cell count was made in the accepted clinical manner using a Levy hemocytometer.

Red blood cell length

Cell lengths were determined from air-dried blood smears which were stained five minutes with Wright's blood stain. Random microscopic fields were brought into focus and all cells that would aline properly, as the micrometer was revolved through a 180° arc, were measured. This was a slow process as often no cells would be in line in a given field, but since randomness was a desirable factor, this method was faithfully pursued. Cell lengths are reported in microns.

Plasma volume and blood volume

Plasma volume was done by the dye dilution method using Eastman's T-1824 (Evans Blue). The dye was injected directly into the exposed ventricle of an anesthesized fish by means of a 1 ml. tubercular syringe manipulated by a microburet manufactured by the Micro-metric Instrument Company (Figure 2). A number 30

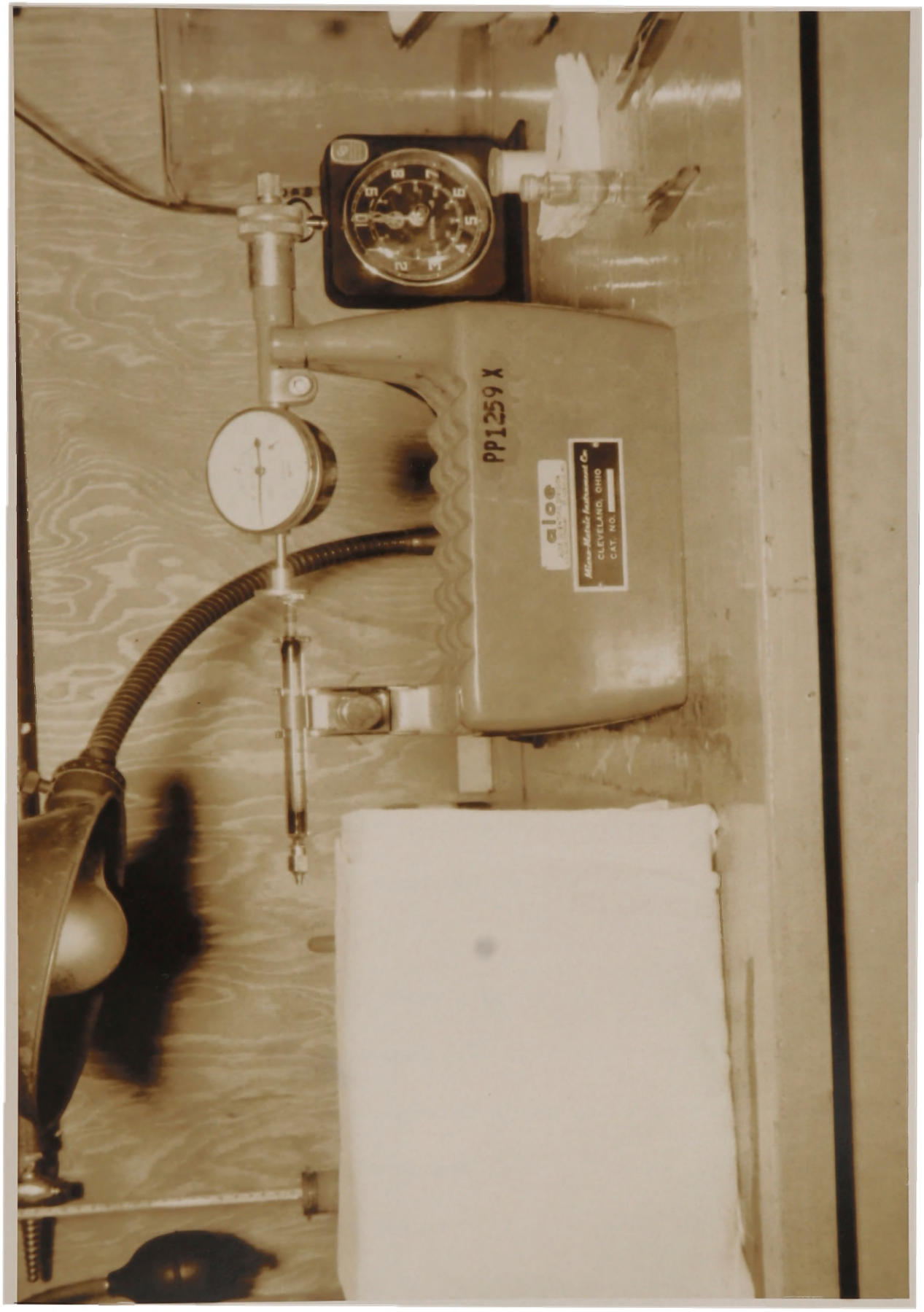

Figure 2. Micro-injection apparatus.

gauge injection needle was needed to introduce the dye with no leakage of blood during or after the injection. Using a 6.0 mg./ml. solution of dye, 0.074 mg. were injected into the fish in a total volume of 0.012 ml. If bleeding due to the incision, or during or after the injection was unusually prevalent, the fish was discarded. Of course, there was always a small amount of seepage of blood, but due to the fast coagulation time of fish blood--a matter of a few seconds--this was at a minimum. It was felt that to counteract this error due to bleeding, the volume of dye, 0.012 ml., would not be subtracted from the final results. The needle was not withdrawn from the ventricle until all visible concentration of dye was removed from the heart. This prevented leakage of the dye, and also helped seal the puncture quickly due to the action of undiluted blood. It was found best to remove the needle at the peak of systole or beginning of diastole, so the blood would not be squeezed out of the fresh hole.

The injected fish were placed on their backs in a V-shaped trough lined with wet gauze, and artificial respiration was induced by means of water flowing over their gills. (Figure 3). Water from an aquarium reservoir was led to the fish by means of rubber tubing and a glass tube which was placed in the fish's mouth. Water flowed at the rate of 150 drops per minute. The fish, unless

Figure 3. Artificial respiration apparatus.

it was deeply anesthesized, would pump the water over the gills with relatively normal opercular movements. If opercular movements were absent, slight stimulation on the ventrad of the mouth would cause a spasmodic movement and hasten the return of the respiratory reflexes.

The fish would remain in this position for ten minutes, although they would usually have to be removed to be reanesthesized at least once during the period. Ten minutes was considered sufficient time to complete the entire mixing of dye even though the circulation time of the fish was unknown. The calculated circulation time of Opsanus tau was two minutes (Brown, 1957), which, if the trout were approximately equal, would indicate five complete circulations of the dye within the period allowed.

At the end of the ten minutes blood was drawn from the fish as described above. The blood was then expressed into a 1 ml. centrifuge tube from which a hematocrit sample was removed. The remaining blood was centrifuged at 2500 RPM for ten minutes. The plasma was diluted by adding 40 cmm. of it to 0.5 ml. of distilled water in a cuvette. This dilution was read in the Spectronic 20 at a wavelength of 650 mμ. The concentration of the dye in the undiluted plasma was then determined from a standard curve made previously by reading known dilutions in the colorimeter.

The accuracy of this test is unknown since two or more determinations could not be made on the same fish, and there are no known data of this type for this species which could be compared.

Splenectomies

Fish to be spelenectomized were anesthesized as previously described, placed in the V-shaped trough and administered artificial respiration. A small incision, about 1.5 cm. long, was made by means of a pointed scalpel in the ventral abdomen slightly posterior to a midpoint between the pectoral and pelvic girdles. The spleen would usually lie beneath this location. By means of a pointed forceps, the spleen was withdrawn through the incision and cut off. It was found that the vessels serving the spleen were small, and there was no need of tying them off. With the spleen removed, the incision was closed with one suture, and the area dried with a sponge and covered with collodion. By this time the fish would be out of the anesthetic enough to return it to an aquarium. This operation did not appear to unduly harm the fish. Fifteen minutes after being returned to the tank they showed no signs of abnormal behavior and would feed on trout pellets.

Body water

Total body water, in grams per 100 grams of body weight, was determined on fish whose liver, gall bladder, spleen, kidney, and immature gonads were removed. The fish were weighed to the nearest tenth of a gram and dried in an oven 95° C. to 105° C. for 48 hours. They were cooled in a desiccator and again weighed, the loss in weight being considered as total body water.

The main objection to the method would be the fact that the organs were removed previous to the determination. The greatest error introduced by this procedure would still be small. Using the mean weight of the fish without organs as 15.6 grams and the average body water weight as 12.2 grams, it can be determined that the water would constitute 78 percent of the body weight. By adding the weight of the excised organs, the mean weight of the wet fish becomes 16.0 grams; and assuming the organs to be 90 to 100 percent water--an extreme estimate--the total body water would become 79 percent of the body weight. This means the error introduced by removal of these organs would be 1.4 percent at the very most.

Organ weights

Freshly removed organs were weighed on a Roller-Smith balance and the results reported in milligrams.

RESULTS AND DISCUSSION OF PHYSIOLOGICAL VALUES OF THE NORMAL HATCHERY-RAISED RAINBOW TROUT

The paucity of physiological data pertaining to fish in general, and rainbow trout in particular, made it imperative to establish normal physiological values through the use of large control groups. To obtain this physiological data from small fish, it was necessary to drastically adapt methods employed in mammalian physiology to the problems encountered. These modifications are presented in the extensive and detailed section entitled "Materials and Methods.

The data and their statistical evaluation pertaining to this section can be found in Appendix II, Part A.

Organ Weights

The analysis of these data on the kidney, liver, and spleen was approached in the following ways: (1) as a linear regression of organ weight on body weight, and (2) organ weights expressed as percent of body weight.

All regression and correlation coefficients were statistically tested, and they proved to be highly significant. The straight lines,

fitted by the least squares method, verifying this correlation of organ weight on body weight are illustrated in Figure 4, for the kidney; Figure 5, for the liver; and Figure 6, for the spleen.

Thus, having proved the premise that organ weights are directly related to body weight, the prediction equations, including the standard error of the estimate, can be stated as follows:

Kidney:

$$Y \pm 20.43 \text{ mg.} = -10.50 + 8.75 \times \text{grams}$$

Liver:

$$Y \pm 40.46 \text{ mg.} = -38.93 + 12.89 \times \text{grams}$$

Spleen:

$$Y \pm 11.53 \text{ mg.} = -7.93 + 2.97 \times \text{grams}$$

$\underline{Y}$ is the predicted weight of the organ and $\underline{x}$ is the given body weight.

There is no evidence that extrapolation of the data beyond the range determined experimentally would be valid; therefore, the use of these equations beyond the range of the graphs should be questioned.

When expressed in terms of organ weight as a percentage of body weight, the means of these data collected on ten fish are as follows:

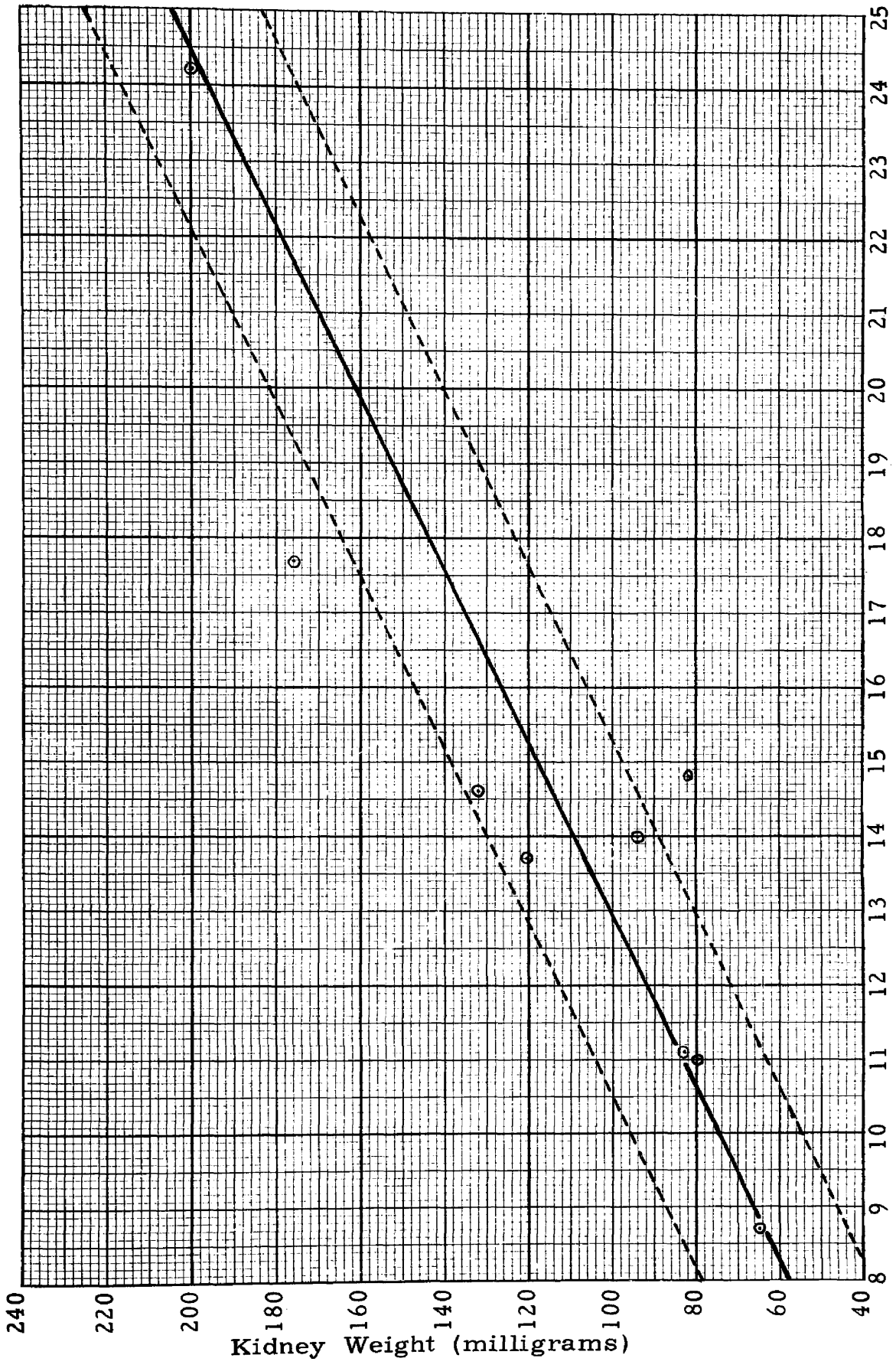

Figure 4. Normal kidney weights.
(Dotted lines indicate standard error of the estimate.)

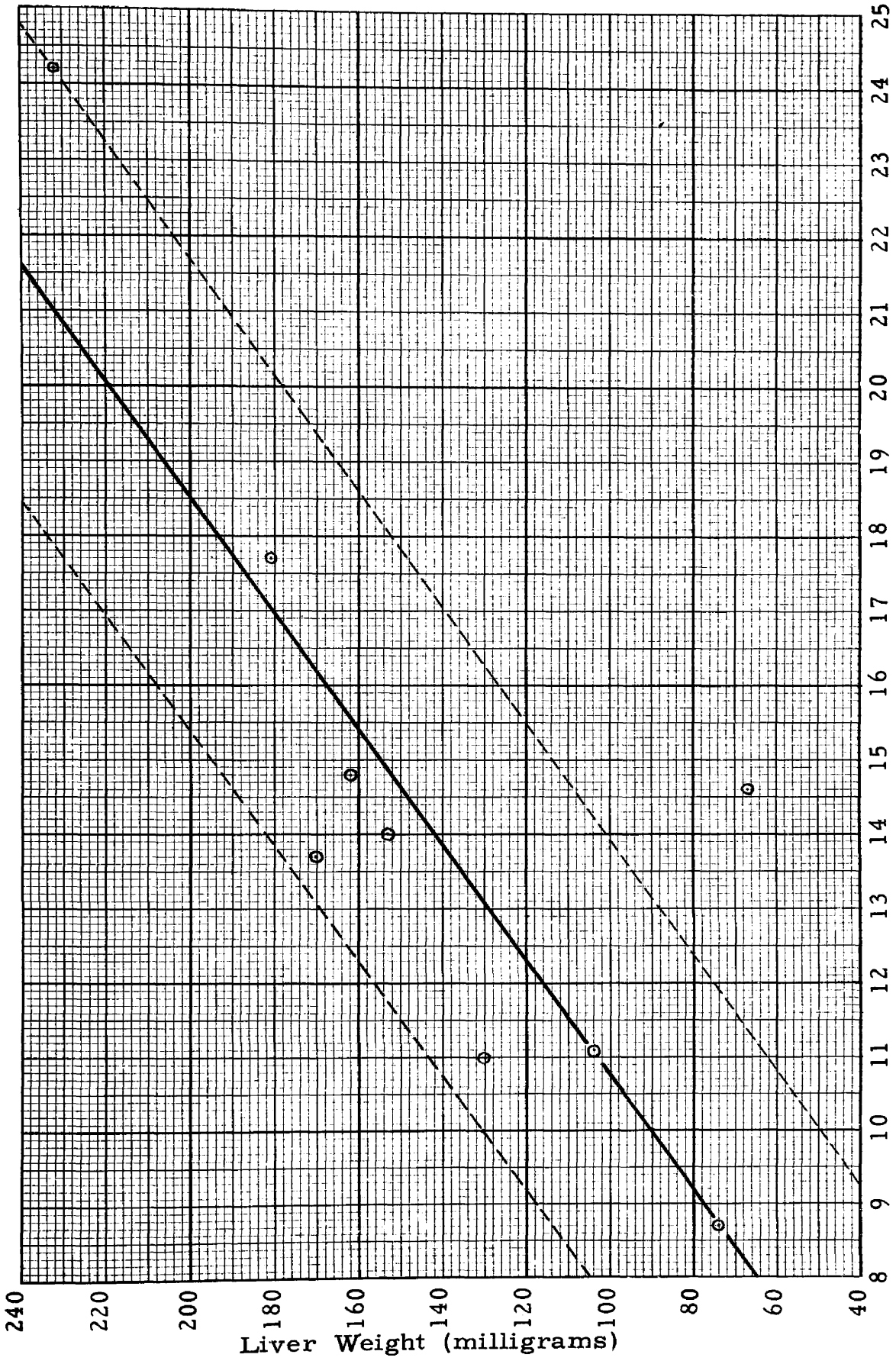

Figure 5. Normal liver weights.
(Dotted lines indicate standard error of the estimate.)

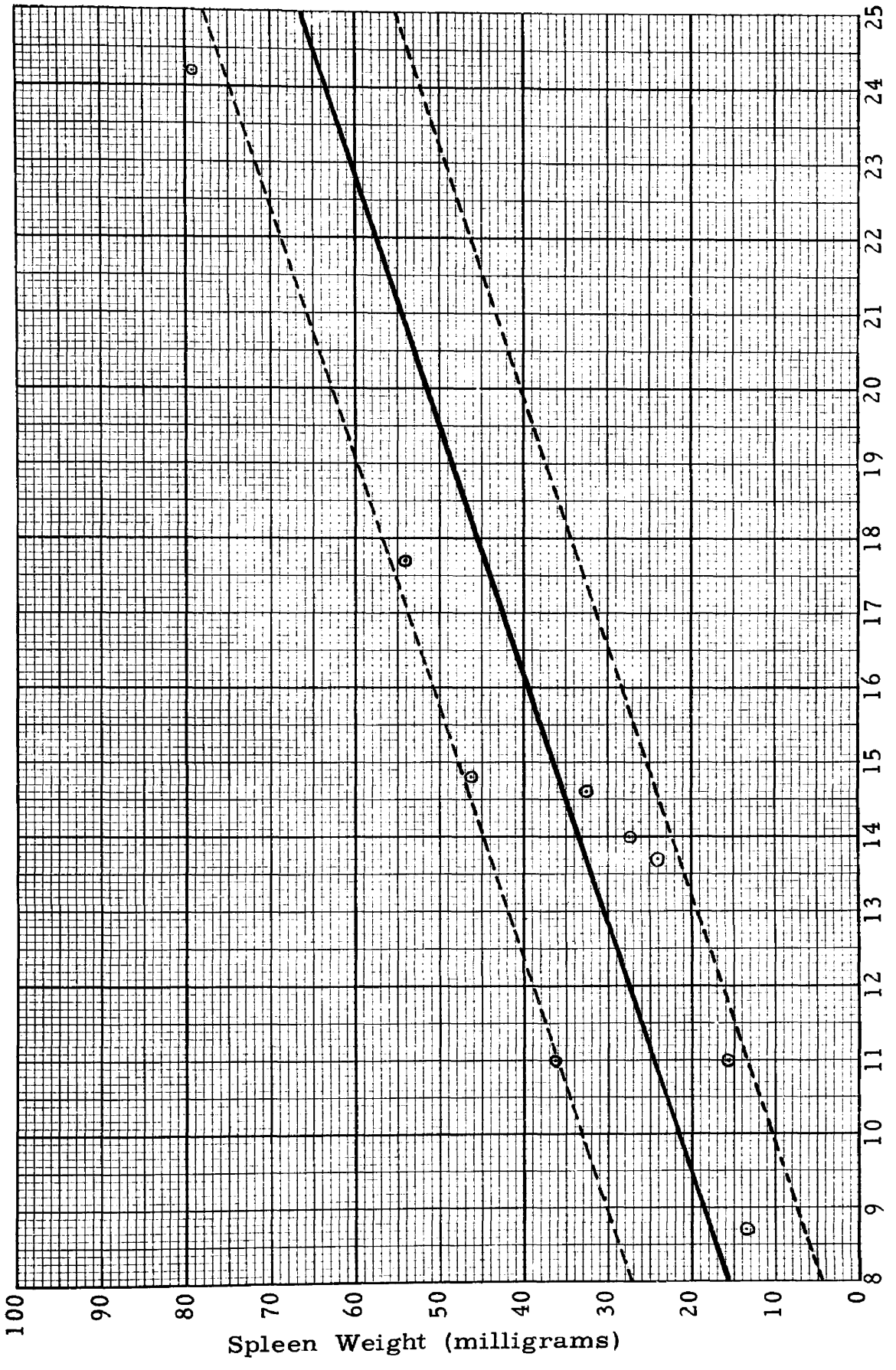

Figure 6. Normal spleen weights.
(Dotted lines indicate standard error of the estimate.)

Kidney:

0.77 g./100 g. of body weight ± the standard error of 0.0034 g./100 g.

Liver:

1.02 g./100 g. of body weight ± the standard error of 0.08 g./100 g.

Spleen:

0.24 g./100 g. of body weight ± the standard error of 0.023 g./100 g.

No information on organ weights pertaining to rainbow trout or members of the family Salmonidae was found in the literature, although Krumholz (1956) reported data for eight species of warm water fish which had been exposed to low level radioactive wastes. His data can be found in Table 8 of this section.

Krumholz's results on the kidney range from 0.11 to 0.98 percent, and on the liver from 0.84 to 3.65 percent. The results presented herein fall well within the ranges for these organs. The data on spleen weight reported by Krumholz ranged from 0.06 to 0.14 percent, which is well below that found for the trout, 0.24 percent.

There are two points that should be emphasized in this comparison of data. First, there are definitely species differences

TABLE 8

AVERAGE PERCENTAGE OF TOTAL WEIGHT OF STABLE TISSUES CONTRIBUTED BY EACH TISSUE DISSECTED FROM INDIVIDUAL OF EIGHT SPECIES OF FISH FROM WHITE OAK LAKE
(Krumholz, 1956)

Fish	Kidney	Liver	Spleen
Bluegill	0.24	1.19	0.11
Black Crappie	0.11	1.03	0.10
White Crappie	0.16	1.17	0.06
Largemouth Bass	0.20	0.84	0.08
Carp	0.59	3.65	0.13
Bullhead	0.98	2.25	0.05
Redhorse	0.57	2.00	0.11
Gizzard Shad	0.78	1.90	0.14

indicated by Krumholz's data on warm water fish. The trout, a cold water species, might easily differ to a greater extent, as evidenced by the spleen weight. Secondly, the fact that the fish under investigation by Krumholz were exposed for long periods of time to radioactivity must be recognized.

Data were obtained on gall bladder and gonad weights, although they were found to be too variable to be of use in this work. The gall bladder was weighed full of bile, and its weight reflected the secretion and expulsion of bile which are variables that may or may not be logically correlated with body weight. Gonad weights were extremely variable in these young fish due to their being in various stages of sexual development. Both the gall bladder and gonad results are presented in Appendix II.

Total Body Water

These data, obtained from ten fish, are handled in the same manner as the organ weights. Once again the regression and correlation coefficients proved to be highly significant, and the following regression equation was calculated:

$$Y \pm 0.47 \text{ g.} = -0.62 + 0.82\ x$$

Figure 7 illustrates this correlation between total body water on body weight.

When expressed in terms of percent of body weight, the mean of the results determined on ten fish is:

77.4 g./100 g. of body weight ± the standard error of 1.02 g./100 g.

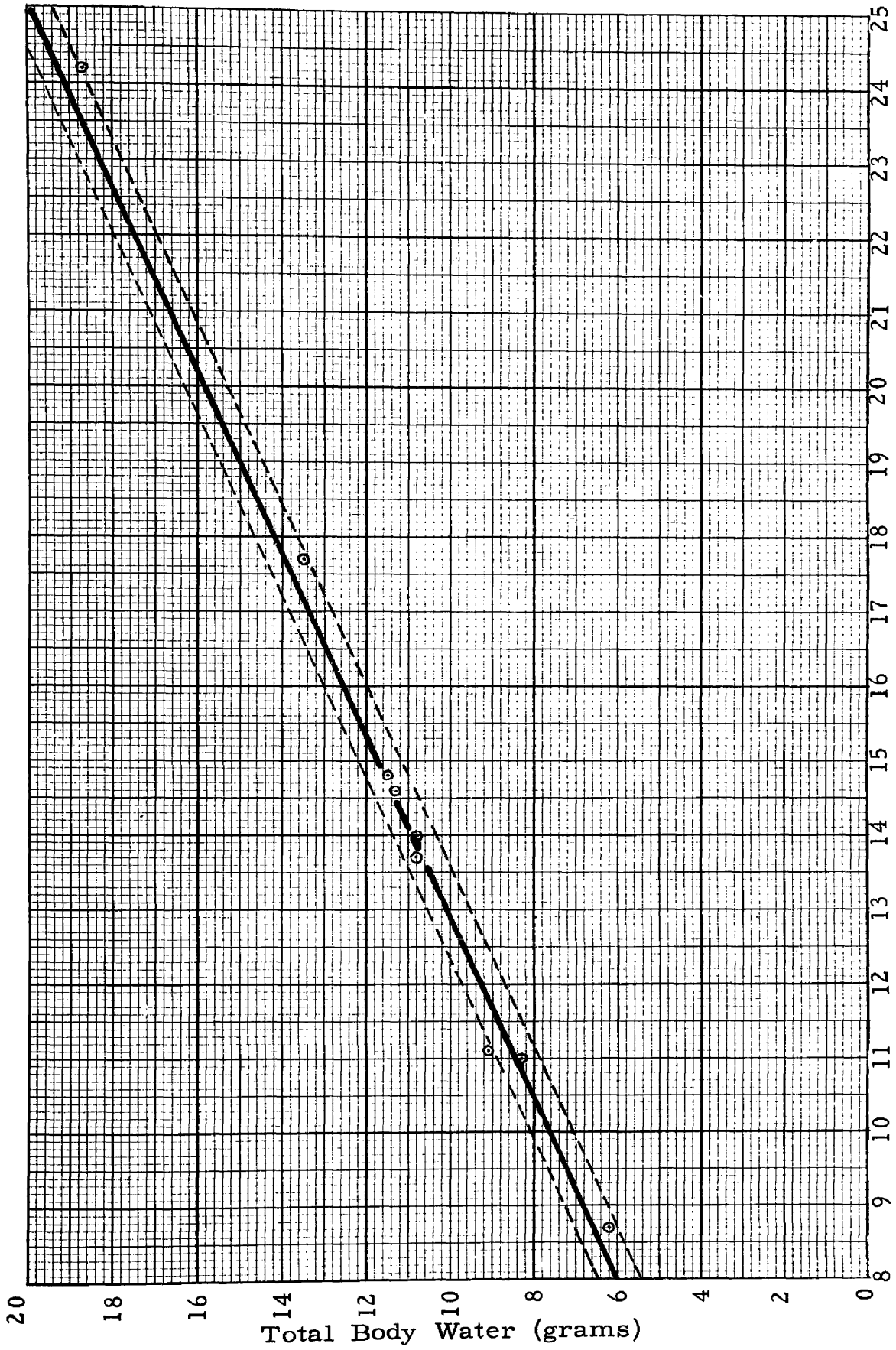

Figure 7. Body weight plotted against body water.
(Dotted lines indicate standard error of the estimate.)

Brown (1957), in her recent compilation of published work, states that the results of water determinations are difficult to compare due to the widely differing techniques used by various authors. After a complete review of the literature, she came to the conclusion that "fish in general contain about 80 to 85% water, extreme values ranging from 53 to 89.3%." These data on the trout, although below 80 percent, still fall well within the range.

Hematocrits

Hematocrits were found to be 31.8 ± a standard error of 1.39 ml. of red blood cells per 100 ml. of blood. Spector (1956), in his Handbook of Biological Data, a complete review of all literature, reported an average hematocrit for rainbow trout of 27.2 ml. R.B.C./100 ml. of blood with a range in data between 22 ml. to 36 ml./100 ml.

Hemoglobin

The mean of the hemoglobin determinations on ten fish were found to have a value of 6.51 ± the standard error of 1.27 g./100 ml. of blood. Spector (1956) reports for trout a hemoglobin value of 8.5 g./100 ml. with a range of 6.2 g. to 11.5 g./100 ml. Prosser

et al. (1950) gives a range from 8.7 g. to 17.6 g./100 ml. for fish in general.

The data reported in this work fall in the lower part of the range reported by Spector (1956). This may be due in part to the general adoption of mammalian techniques without the adaptations necessary for nucleated blood. The usual methods call for a colorimetric analysis of hemolyzed blood at some point during the procedure. If the interfering nuclei are not removed prior to this manipulation, the results will be high. The author made a few determinations of hemoglobin on nucleated samples and found these values agreed quite well with the upper range published by Spector. They also agreed very well with Black's (1955) values of 11.0 g./ 100 ml.

Red Blood Cell Counts

The red blood cell counts on ten fish averaged 1.11 ± 0.098 million cells/cmm. This agrees very well with Spector's (1956) value for trout of 1.01 million cells/cmm. with a range of 0.74 to 1.5 million cells/cmm. Prosser et al. (1950) reports a range for fish in general of 0.585 to 2.685 million/cmm.

Red Blood Cell Lengths

By taking an average length of ten cells/fish and using ten fish, a value of 14.69 μ $\pm$ the standard error of 0.24 was determined. Prosser et al. (1950) gives a value of 16.7 μ for trout. It is interesting to note at this point that Brown (1957) published data on the eel, 14.6 μ in length and 1.10 million cells/cmm. in number, which agree closely with the trout data reported here. This is, of course, no more than an interesting observation.

Plasma Volume and Blood Volume

The regression and correlation coefficients relating plasma and blood volume to total body weight were found to be highly significant. The following prediction equations, including the standard error of the estimate, were calculated:

Plasma volume:

$$Y \pm 0.07 \text{ ml.} = 0.094 + 0.0154 \times \text{grams}$$

Blood volume:

$$Y \pm 0.08 \text{ ml.} = \overset{0.15}{\cancel{0.050}} + 0.023 \times \text{grams}$$

When expressed in milliliters per 100 grams of body weight, the mean of ten determinations is:

Plasma volume:

2.13 ml./100 g. ± the standard error of 0.14 ml./100 g.

Blood volume:

3.25 ml./100 g. ± the standard error of 0.16 ml./100 g.

Although no plasma or blood volume data specifically related to trout could be found in the literature, Prosser et al. (1950) reported a blood volume for teleost fishes in general of 1.5 to 3 percent of body weight. Brown (1957), in her compilation of data on Actinopterygii, reported a range of 1.4 to 2.8 percent of the body weights.

Various workers have all reported blood volume values lower than those observed by this author. The most plausible explanation of this discrepancy is that, until this report, blood volume experiments had been performed on large fish, all over 100 grams. Also, specific averages were calculated from small numbers of fish, usually one, two, or three. There is no evidence that young fish, such as used in this study, have a larger blood volume than adult fish, although this possibility must be considered in viewing these data. In addition, the methods used to obtain the published results vary from direct washing to dilution techniques employing a variety of vital dyes. Further, since species differences have been indicated in other physiological data, there is reason to believe this

may be a cause of the discrepancy between this work and the published data.

RESULTS AND DISCUSSION OF POTASSIUM CHROMATE-INDUCED CHANGES IN THE PHYSIOLOGICAL VALUES OF HATCHERY-RAISED TROUT

Median Tolerance Limit

To establish a working concentration of potassium chromate, expressed in terms of the hexavalent chromium ion, a twenty-four hour median tolerance limit (TLm) was determined. Figure 8 illustrates the linear interpolation of the bioassay data with a resulting twenty-four hour TLm of 100 mg. Cr/l. Grindley (1946) reports a toxicity to rainbow trout using potassium chromate, but his results are such that they are impossible to interpret in light of this experiment. Fromm and Schiffman (1958) found a TLm for largemouth bass of 195 mg. Cr/l. using potassium chromate. Oshima (1931) found that young eels would survive in concentrations of potassium chromate equal to 520 mg. Cr/l. for an average of 12.4 hours. We can conclude from the above that trout, an intolerant species, are more susceptible to the toxic effects of potassium chromium than eel or largemouth bass.

The bioassay produced a level at which 50 percent of the trout die within twenty-four hours; but since this work is primarily

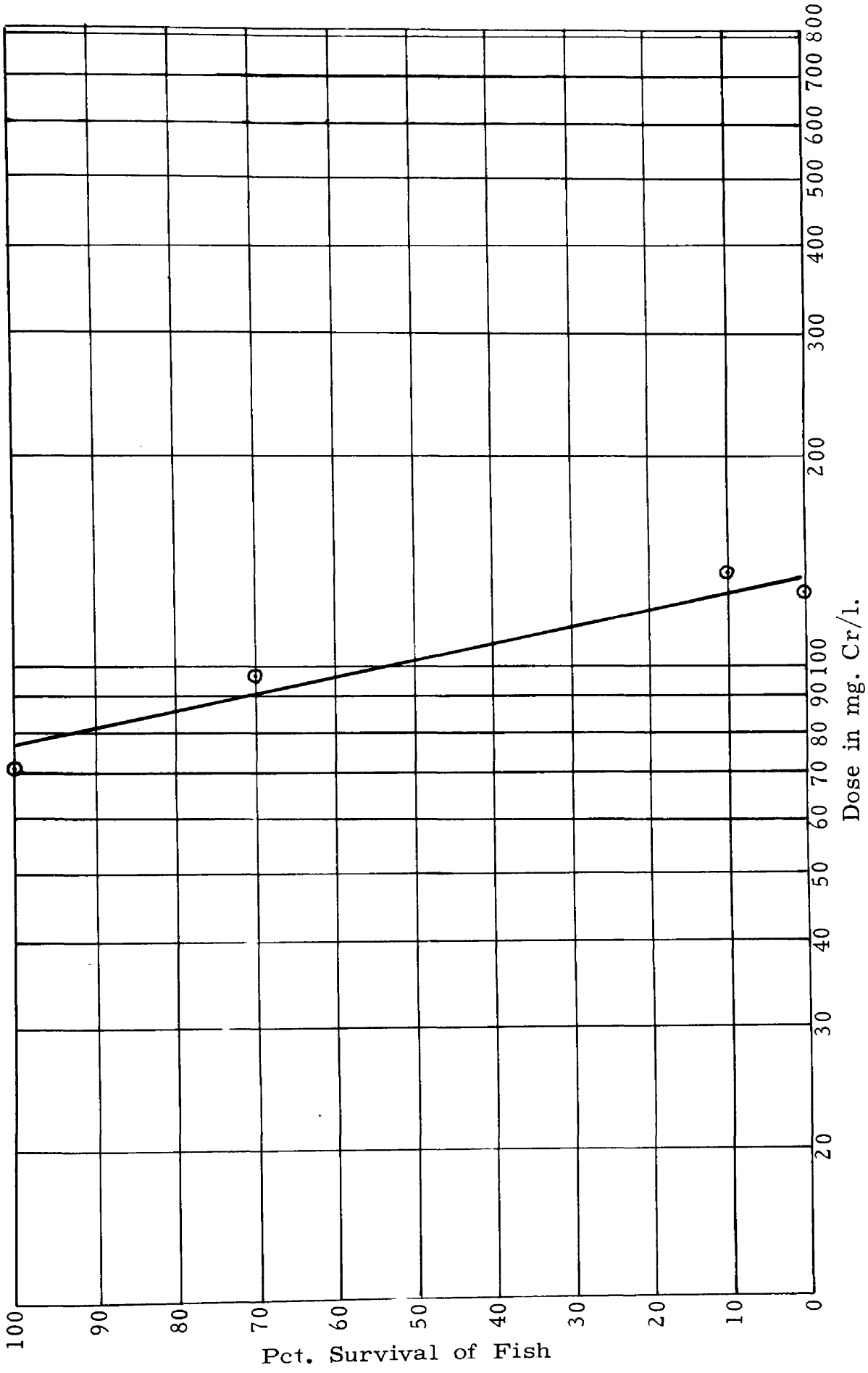

Figure 8. Estimation of 24 hour median tolerance limit by straight-line graphical interpolation.

aimed at levels of chronic toxicity, a concentration of 20 percent of the TLm, or 20 mg. Cr/l. was arbitrarily chosen.

Tracer Experiments

Once the working level was established, tracer experiments were done to find the target tissues. Aquaria were set up using both the stable chromium and the radioactive Cr^{51}, so that each milliliter of water contained 18.6 µg of chromium with an activity of twelve counts per minute. Three fish were exposed to this concentration for twenty-four hours, after which they were sacrificed, and the spleen, liver, gall bladder, and a section of the caudal peduncle were analyzed. The caudal peduncle was used as a control, since it is predominantly muscle tissue; thus, there was no reason to suspect it to concentrate the chromium.

Expressed in terms of micrograms of chromium/100 mg. of tissue and as a mean count per minute/100 grams of tissue, the results are shown in Table 9.

Analysis of variance methods showed that the spleen and gall bladder were significantly more active than the caudal peduncle, whereas the kidney and liver did not differ from the control.

Based upon the above information, the path of the chromium ion through the fish was postulated. The chromium ion, having

TABLE 9

RESULTS OF TRACER EXPERIMENTS

Organs	Mean cpm./100 mg. of Tissue	Cr/100 mg. Tissue (μg.)
Spleen	34.92	60
Gall bladder	33.24	57
Kidney	13.88	23
Liver	8.28	14
Caudal peduncle	4.83	8

gained entrance to the blood by an unknown means, is distributed throughout the animal and tends to be concentrated in the spleen. The red blood cell of mammals is known to have an intense affinity for hexavalent chromium and Cr^{51} labeled cells have been used in blood volume determinations. This means of transport by the blood of fish seemed to be highly probable in light of this experiment.

Although the function of the spleen of fish is unknown, based upon knowledge of mammalian spleens, it can be reasoned that the reticulo-endothelial system of the spleen could possibly cause a

breakdown of the chromium-laden cells with a release of chromium products into the efferent splenic vessel which leads directly to the liver. The liver could then eliminate this chromium product by way of the bile. Although there was no evidence that the liver concentrated chromium above the level found in the nonspecific muscle tissue, the gall bladder, including the bile, implicated this organ as a path of elimination of chromium. Fromm and Schiffman (1958) theorized that it was this chromium-laden bile which caused the extensive intestinal damage that was observed in the largemouth bass.

Although the kidney was not associated with chromium removal by evidence gathered in this experiment, neither did the evidence omit it as a pathway. Possibly, like the liver, it is an active route, but does not concentrate the chromium within its tissues. Analysis of urine could be done to enlighten this hypothesis.

Organ Weights and Total Body Water Experiments

Thus, having definitely implicated the spleen and liver and possibly the kidney, studies on these organs and the total body water were done to demonstrate changes in the weight of these organs and changes in water composition due to exposure to approximately 20 mg. Cr/l. It was found in comparing the data from five fish so exposed with the regression lines of the normal rainbow trout,

that no differences could be detected under the experimental conditions.

Results of Blood Experiments

Hematocrits were determined on four of the above five fish, and the results averaged 54 ml./100 ml. as compared to the 31.8 ml./100 ml. of the normal trout. In light of the foregoing, it was decided to study the blood in general and the causes for the rise in hematocrit in particular. Since the mamalian spleen acts as a reservoir for red blood cells, releasing those cells in time of stress, a study of the splenic relationships to the hematocrit was in order. The following experiment was designed to answer the questions posed above.

Intact and splenectomized fish were kept in tap water for twenty-four hours. This was done to allow operated fish to recuperate. After this twenty-four hour period (1) ten normal fish were exposed to approximately 20 mg. Cr/l. (normal chromium group); (2) ten splenectomized fish were kept in tap water (splenectomized control group); and (3) ten splenectomized controls were exposed to approximately 20 mg. Cr/l. (spelenectomized chromium group). Samples were obtained for all three groups after twenty-four hours for the following determinations: (1) length, (2) weight,

(3) hematocrit, (4) red blood cell count, (5) hemoglobin, (6) plasma volume, and (7) blood volume. Red blood cell length determinations were not done on splenectomized fish. The results of the experiments are shown in Table 10.

Plasma and blood volumes were expressed as a linear regression of volume on body weight. Once again, all regression and correlation coefficients proved to be highly significant. The equations for the regression lines, including the standard error of the estimate, are as follows:

Normal chromium

Plasma volume:

$$Y \pm 0.116 \text{ ml.} = 0.16 \times \text{grams}$$

Blood volume:

$$Y \pm 0.116 \text{ ml.} = 0.04 + 0.028 \times \text{grams}$$

Splenectomized control

Plasma volume:

$$Y \pm 0.084 \text{ ml.} = 0.164 + 0.017 \times \text{grams}$$

Blood volume:

$$Y \pm 0.88 \text{ ml.} = 0.14 + 0.032 \times \text{grams}$$

TABLE 10

RESULTS OF BLOOD EXPERIMENTS

Determination	Mean	Standard Error
Normal Fish Exposed to 20 mg. Cr/l.		
Hematocrit	43.8 ml./100 ml.	1.56 ml.
Hemoglobin	6.6 g./100 ml.	0.25 g.
R.B.C. count	1.25 million cell/cmm.	0.032 million
Cell length	14.93 μ	0.15 μ
Plasma volume . . .	1.61 ml./100 g.	0.12 ml.
Blood volume	3.01 ml./100 g.	0.18 ml.
Splenectomized Fish in Tap Water		
Hematocrit	2.85 ml./100 ml.	1.42 ml.
Hemoglobin	5.61 g./100 ml.	1.13 g.
R.B.C. count	1.04 million cell/cmm.	0.18 million
Plasma volume . .	2.44 ml./100 g.	0.12 ml.
Blood volume	3.85 ml./100 g.	0.16 ml.
Splenectomized Fish Exposed to 20 mg. Cr/l.		
Hematocrit	40.6 ml./100 ml.	2.92 ml.
Hemoglobin	7.42 g./100 ml.	0.51 g.
R.B.C. count	1.38 million cell/cmm.	0.11 million
Plasma volume . . .	1.74 ml./100 g.	0.10 ml.
Blood volume	3.16 ml./100 g.	0.14 ml.

Splenectomized chromium

Plasma volume:

$$Y \pm 0.069 \text{ ml.} = 0.05 + 0.015 \times \text{grams}$$

Blood volume:

$$Y \pm 0.077 = 0.18 + 0.022 \times \text{grams}$$

The regression lines for the plasma volume data are plotted in Figure 9, and for the blood volume data in Figure 10.

The results of these experiments were compared among themselves and with the normal trout data. Analysis of variance, covariance, and t-tests were used with the following results.

Hematocrit

The splenectomized and intact animals exposed to chromium had hematocrits significantly higher than those fish, both splenectomized and intact, not exposed to chromium.

Hemoglobin

The splenectomized animals exposed to chromium had a significantly higher hemoglobin content than did those splenectomized fish that were not exposed. There was no evidence of differences between other groups.

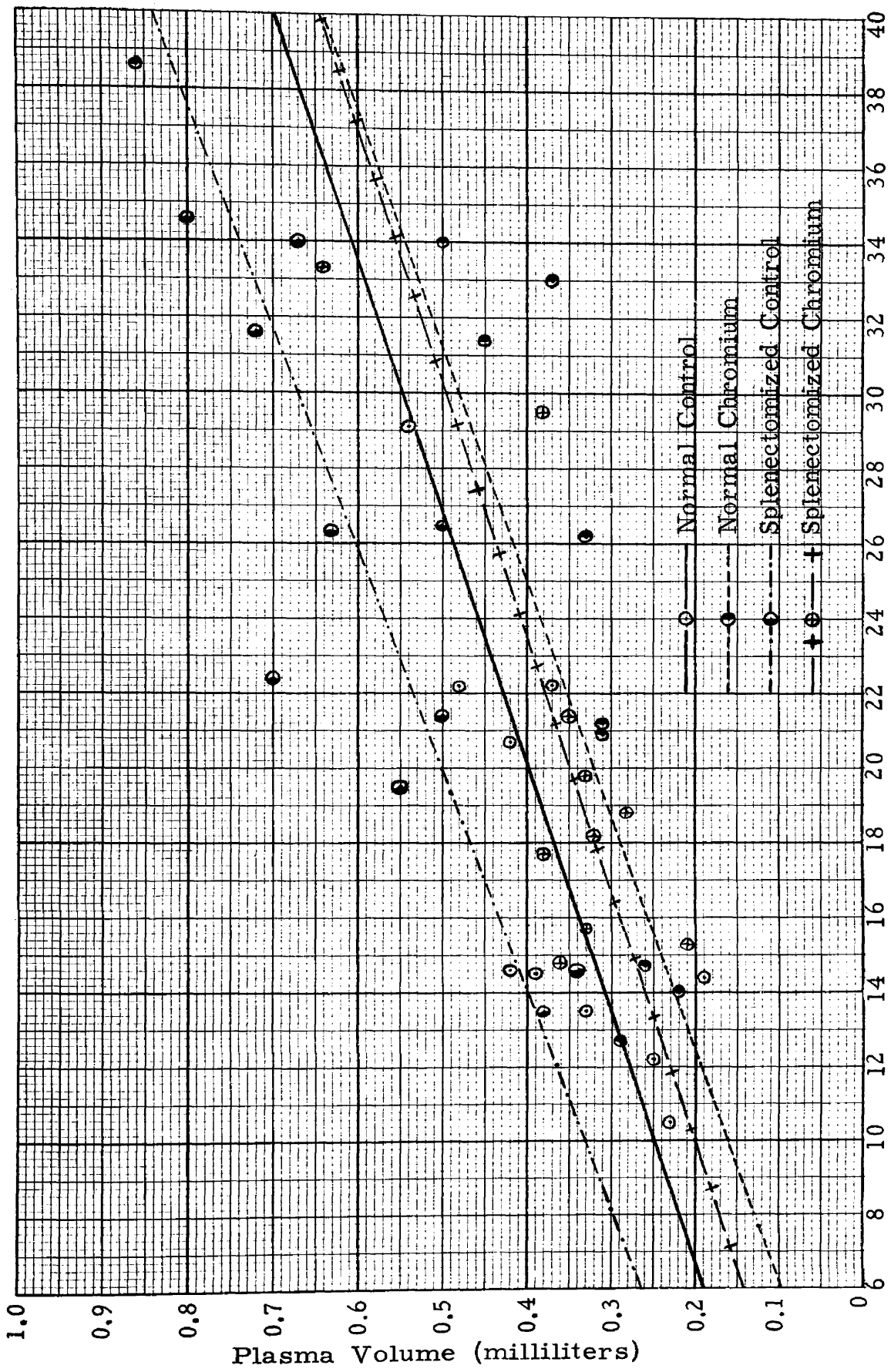

Figure 9. Comparison of plasma volumes.

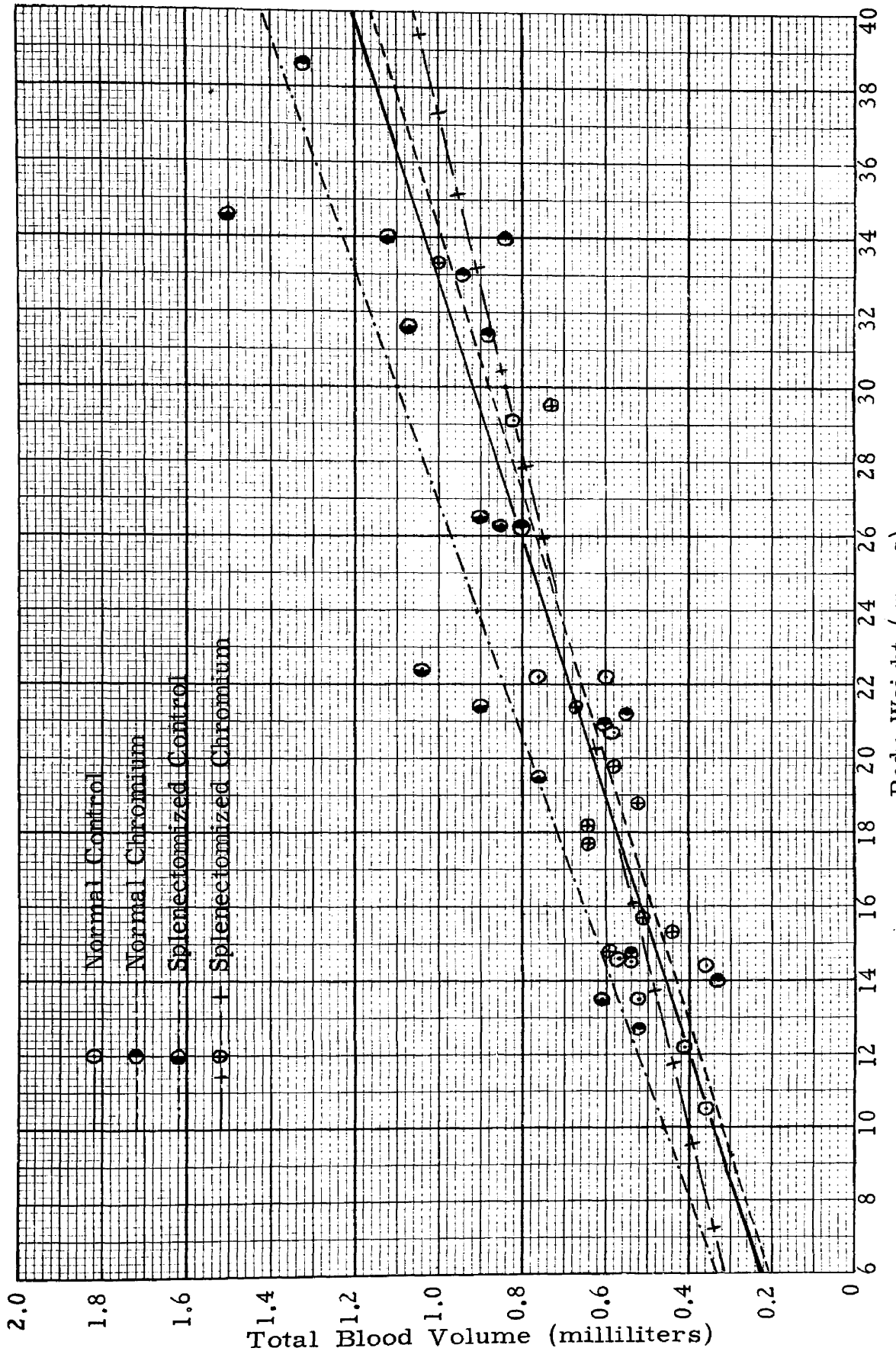

Figure 10. Comparison of total blood volumes.

Red blood cell count

According to the analysis of variance, the splenectomized animals exposed to chromium had significantly higher blood cell counts than the intact and splenectomized controls. By "t" test, the normal animal exposed to chromium also had a higher blood count than the normal count.

Red blood cell length

By "t" test, the normal animal exposed to chromium had significantly larger blood cells than the normal trout in tap water. No measurements were made on splenectomized fish.

Plasma volume and blood volume

By covariance, the splenectomized fish in tap water had a significantly higher plasma and blood volume than the splenectomized fish exposed to chromium. The experimental evidence did not show a difference in the other groups.

Discussion of the Increase in Hematocrit

The highly significant increase in hematocrits of normal trout exposed to chromium could be brought about in three general ways:

(1) by an increase in cell size, (2) by an increase in cell number, and (3) by a decrease in plasma volume. Since there was a highly significant increase in cell size, this matter will be discussed first.

If normal trout have a red blood cell count of 1.11×10^{6} cell/cmm. and a hematocrit of 31.8, then (1) there are 1.11×10^{11} cells/100 ml. of blood and (2) each milliliter of the packed red blood cells contains 3.50×10^{9} cells. In the normal trout exposed to chromium, using the same mathematics, it was found that (1) there are 1.25×10^{11} cell/100 ml., and (2) that each milliliter of the packed red blood cells contains 2.85×10^{9} cells. Assuming that the change in numbers of packed cells was due to change in volume only, the 1.11×10^{11} cells in the normal trout, if changed in volume like those in the chromium-exposed fish, would cause a rise in hematocrit from 31.8 to 38.9. Therefore, the increase in cell volume accounts for 59 percent of the total rise in hematocrit. If the cell number increased by 1.40×10^{10} cell/100 ml. of blood and, as calculated above, there are 2.85×10^{9} cell per milliliter of packed cells, then the increase in number would account for 4.9 milliliters of packed cells, or 41 percent of the increase in hematocrit. Since these percentages were calculated from means, the standard error of these means must be kept in mine when contemplating these results.

When the hematocrit of an animal increases significantly, the blood volume must increase and/or the plasma volume must decrease. Unfortunately, neither of these events could be shown to occur by statistical examination of the blood and plasma volume results. Therefore, although changes must occur, they are too small to be measured by the method used herein.

In light of the above evidence, it can be concluded that approximately 59 percent of the increase in hematocrit due to exposure to potassium chromate is due to an increase in cell volume and approximately 41 percent of the increase is due to increase in number of cells. No evidence of a change in plasma or blood volume could be detected.

Discussion of the Causes of the Red Cell Volume Change

The reasons for the red cell volume change were not considered experimentally in this study. Theoretically, this increase in volume could be caused by a stress reaction such as postulated by Irving, Black, and Safford (1941). They found, using twenty-five rainbow trout, erythrocytes would swell 1 to 21 percent, with an average swelling of 10 percent, when subjected to tensions of carbon dioxide above 10 mm. Hg. The swelling of the red blood cells was

found to be more intense than would be expected if the CO_2 tension was the only variable which determined volume change.

In the chromium experiments herein, carbon dioxide was not a factor. Using Moore's (1939) graphs in connection with the alkalinity and pH of the solutions, carbon dioxide was never detected in measurable amounts.

Another cause of increase in volume could be due to the affinity of the erythrocytes for chromium. If the chromium enters the red cell, the osmotic concentration might be altered sufficiently to cause water to be drawn into the cell. Since the molecular form of the chromium complex in the blood and in the red blood cell is unknown, calculation of the amount of chromium necessary to change the osmotic pressure in such a way as to cause this increase in volume was not undertaken.

Discussion of the Role of the Spleen in the Increase in Cell Number under Potassium Chromate Stress

The mammalian spleen is known to store erythrocytes for release into the circulating blood during times of stress. The splenectomized fish experiments were designed to demonstrate whether the fish spleen added erythrocytes to the circulating blood under potassium chromate stress. The splenectomized fish under

control conditions did not show a difference in hematocrit, hemoglobin, or cell count when compared to normal control fish. The splenectomized fish under potassium chromate stress (20 mg. Cr/l.) did show a significant increase in hematocrits and red cell count above the splenectomized and intact controls. The hemoglobin increased significantly above the level of the splenectomized control fish, but not of that of the intact controls. None of the values determined on chromium-exposed splenectomized fish deviated statistically from those of the chromium-exposed normal fish.

It can be concluded from the foregoing evidence that the presence of the spleen is not necessary to increase the hematocrit under chromium stress, since there was no difference in effects whether the experiments were performed on splenectomized or intact fish.

Discussion of the Part Played by the Potassium Ion in Potassium Chromate Toxicity

The results so far have been expressed in terms of milligrams of chromium ion per liter of water even though the compound used throughout this work was potassium chromate. Since potassium chromate does contain considerable amounts of potassium ion, a further study was undertaken to consider its effect on the foregoing results.

The group of fish used in this experiment was kept in concrete tanks and fed trout pellets prior to the time of its arrival at the laboratory, whereas all other fish used throughout this study were taken from holding ponds; thus, they were dependent on natural food. This fact called for the determination of new control and chromium-exposed values of the hematocrit. In addition, ten fish were exposed to a potassium chloride solution of a concentration equal to 29.8 mg. K/l. The concentration of the potassium ion in the potassium chloride solution was equal to its concentration in the potassium chromate solution. The results are shown in Table 11.

"T" tests failed to show any evidence of a difference between the potassium chloride-exposed fish and the control and chromium-exposed fish. The variance (85.55) of the potassium chloride-exposed samples were significantly higher than the normal samples, and it was assumed that this was an indication of a state of change. It is interesting to note that a sample of fish exposed to 6.6 mg. Cr/l., the lowest concentration of chromium measured that would increase the hematocrit to 43.8 ml./100 ml., also had a highly significant variance (258.75). Perhaps this increase in variance of samples can be expected at concentrations which are just strong enough to cause an increase in hematocrit in only part of the fish. If this assumption is true, then the potassium ion, in addition

TABLE 11

RESULTS OF POTASSIUM CHLORIDE EXPERIMENTS

Solution	Number of Fish	Hematocrit (ml./100 ml.)	Variance
KCl (29.8 mg. K/l.)	10	38.8	85.55
Tap water	10	34.0	13.77
K_2CrO_4 (20 mg. Cr/l.)	10	40.3	24.44

to the chromium, is a potential influence upon the results obtained at potassium chromate concentrations equal to 20 mg. Cr/l.

Whether this potential influence would be an additive effect or a synergistic effect is unknown, but it does add some understanding to the reason why potassium chromate is considered to be more toxic than sodium chromate. This also questions the practice of thinking in terms of chromium ion concentrations when testing such compounds as potassium chromate, especially at the high concentrations involved in acute studies.

The Hematocrit as an Index of Toxicity

Once having demonstrated that the hematocrit increased in fish exposed to potassium chromate, it was then undertaken to determine the lowest concentration of chromium at which this change took place. The information in Table 12, including the results of the normal control group and the normal chromium group, are plotted in Figure 11.

The length of the lines extending above and below the points plotted in Figure 11 are equal to two times the standard error of the mean. By comparing these lines, a rough estimate of statistical differences can be made.

From this graph we can conclude that the hematocrit value is increased at 1.4 mg. Cr/l. and reaches its maximum rise in value at a point between 2 and 4 mg. Cr/l.

This information is interesting to the stream pollution biologist because, although the 24-hour TLm is 100 mg. Cr/l., in reality, concentrations between 2 and 4 mg. Cr/l. cause actual physical change to occur within the trout.

Since the determination of the hematocrit is a simple procedure requiring a minimum of equipment, it would be well worth the investigation to determine whether this rise in hematocrit could

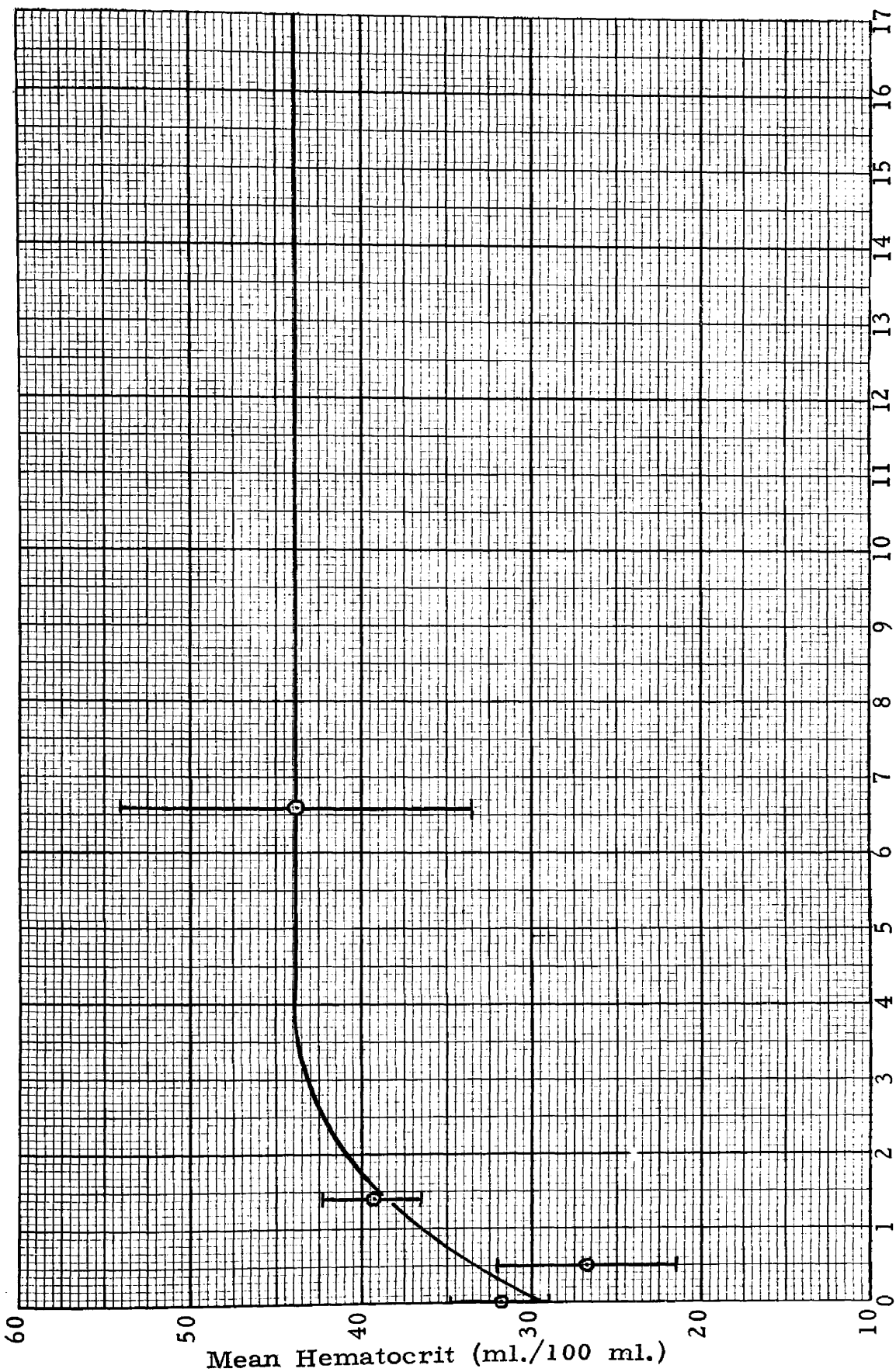

Figure 11. Increase in hematocrit in relation to potassium chromate concentration.

TABLE 12

THE MEAN HEMATOCRITS OF FISH EXPOSED TO VARIOUS CONCENTRATIONS OF POTASSIUM CHROMATE

Number of Fish	Chromium (mg./l.)	Mean Hematocrit (ml./100 ml.)	Standard Error
10	18.0	43.8	1.56
9	6.6	43.8	5.08
10	1.4	39.3	1.47
8	0.5	26.7	2.67
10	0.0	31.8	1.39

be used as an index of chronic toxicity in much the same way as the TLm is used as an index of acute toxicity. This would require experiments which would determine whether an increased hematocrit was a general response to nonspecific stress, or a specific response to potassium chromate.

As stated earlier in this paper, this project does not attempt to answer all of the problems encountered in stream pollution control, but rather, points the way to a better understanding of them.

Spleen Function

During the course of the experiments concerned with blood studies, two series of hematocrits were taken on each group of fish (see Table 13), one series in connection with the blood volume determinations and one series with the other blood tests. The differences in the methods of running these two series were: (1) the blood for the hematocrits and other blood tests was drawn immediately after the fish was anesthesized, and (2) the blood for the hematocrits and blood volume tests was drawn ten minutes after the fish was anesthesized and after 0.012 ml. of 6.0 mg. T-1824/ml. was injected. Designating those hematocrits taken immediately as hematocrits at time $_0$, and those taken after ten minutes as hematocrits at time $_{10}$, the average results are tabulated in Table 13.

When the highly significant difference between the two splenectomized control hematocrits was noticed, it was decided to eliminate the possibility of the T-1824 and the operative procedure as a possible cause of this increase during the ten minute period. Those splenectomized fish in which no T-1824 was injected during the ten minute period still showed a significant increase in hematocrit, while those that were sham operated showed no evidence of an increase.

TABLE 13

RESULTS OF EXPERIMENTS PERFORMED TO INVESTIGATE SPLEEN FUNCTION

Group	Hematocrits (time $_0$)	Hematocrits (time $_{10}$)	Difference
Splenectomized control.	28.5	36.6	8.1
Splenectomized chromium	40.6	44.9	4.3
Splenectomized control no. T-1824	28.5	34.9	6.4
Normal control	31.8	35.0	3.2
Normal chromium . . .	43.8	46.3	2.5
Sham operated control	33.6	35.8	2.2

The above evidence indicates that the spleen of trout acts as a buffer to sudden hemoconcentration. This function is limited, as evidenced by the tendency of the hematocrit to increase in every instance even though this increase cannot be verified by statistical methods. Other evidence of the limited nature of this function is the significant increase in hematocrit in both intact and splenectomized fish exposed to chromium, which would indicate the complete

overwhelming of the buffer capacity of the spleen due to prolonged potassium chromate stress.

More evidence, gathered from experiments specifically designed to investigate the tendency of the spleen to act as a buffer, is needed before definite conclusions can be made. Since this work was primarily aimed at answers to toxicity investigations, and since this limited buffer action of the spleen did not influence the results under chromium stress, further investigation into splenic function was not undertaken.

CONCLUSIONS

1. The following physiological values have been established for the normal hatchery-raised rainbow trout (Table 14):

TABLE 14

PHYSIOLOGICAL VALUES OF THE NORMAL HATCHERY-RAISED RAINBOW TROUT

Determination	Mean Value	Standard Error
Hematocrit	31.8 ml./100 ml.	1.39 ml.
Erythrocyte count	1.11 million cell/cmm.	0.098 million
Hemoglobin	6.51 g./100 g.	1.27 g.
Erythrocyte length . .	14.69 μ	0.24 μ
Plasma volume	2.13 ml./100 g.	0.14 ml.
Blood volume	3.25 ml./100 g.	0.16 ml.
Kidney weight	0.77 g./100 g.	0.039 g.
Liver weight	1.02 g./100 g.	0.077 g.
Spleen weight	0.24 g./100 g.	0.023 g.
Total body water	77.4 g./100 g.	1.02 g.

2. Kidney, liver, and spleen weight; and total body water and plasma and blood volumes were found to correlate highly significantly with body weight.

3. The 24 hour median tolerance limit of trout to potassium chromate in a concentration expressed in mg. Cr/l. is 100 mg. Cr/l.

4. Tracer experiments indicated that the spleen and the gall bladder, including the bile, accumulated chromium above the level of a section of the caudal peduncle, whereas there was no evidence that kidney or liver did.

5. There was no evidence that the weights of the kidney, liver, spleen, and total body water of fish exposed to potassium chromate were different from those of fish in tap water.

6. The hematocrits of both splenectomized and intact fish were significantly higher than control fish in tap water. This rise in hematocrit was found to be due to an increase in cell number and cell volume.

7. There was no evidence that the plasma or blood volume increased or decreased in fish exposed to potassium chromate.

8. The presence of the spleen is not necessary to increase the hematocrit under chromium stress, since there was no difference in effects whether the experiments were performed on splenectomized or intact fish.

9. It was assumed, on the basis of experimental data, that the potassium ion, in addition to the chromium, is a potential influence upon the results obtained at potassium chromate concentrations equal to 20 mg. Cr/l.

10. It was found that the hematocrit reaches its maximum rise in value at a point between 2 and 4 mg. Cr/l. It is hoped that future experimentation might prove that the increase in hematocrit is an index of chronic toxicity.

11. Data indicated that the spleen may act as a limited buffer mechanism to sudden hemoconcentration.

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APPENDIX I

REVIEW OF TOXICITY LITERATURE

Abegg (1950), using bluegill sunfish, Lepomis macrochirus, weighing 20 to 35 grams, tested both Na_2CrO_4 and $Na_2Cr_2O_7$. Under conditions as set forth by Hart, Doudoroff, and Greenbank (1945), Abegg used water with a total alkalinity as $CaCO_3$ of 60 to 120 mg./l., total hardness as $CaCO_3$ of 75 to 150 mg./l., sulphates as $SO_4^=$ of 20 to 50 mg./l. and maximum dissolved solids of less than 500 mg./l. He reported this water not wholly satisfactory as a dilution water for studies pertaining to the effects of chemical compounds on the physiology of fishes.

The fish were tested in 5 gallon round glass battery jars. Each jar contained 12 liters of solution and was kept at 22° ± 0.2° C. by a constant temperature bath. Using aeration Abegg tested six fish in each container. He reported his data in 24 hour median tolerance limits (TLm); that is, concentrations lethal to 50 percent of test animals in 24 hours, and found them to be 930 mg. Na_2CrO_4/l. and 728 mg. $Na_2Cr_2O_7$/l. These test solutions had the following characteristics:

Chemical	Mols./l.	Cal. Osmotic Pressure in Liter Atmos.	Specific Resistance (ohms at 22.2° C.)	pH
Na_2CrO_4	0.0073	0.19	520	8.1
$Na_2Cr_2O_7$	0.0044	0.12	105	5.9

Jones (1939) did work on a series of metallic ions which were added to very soft tap water "of a high degree of purity." The calcium ion concentration was about 1 mg./l. The fish were sticklebacks, Gasterostens aculeatus, usually 30 to 50 mm. in length. Four to five fish were exposed in 2 liters of each test solution, and the solutions renewed at daily intervals. A series of ten to twenty different concentrations of each salt was tested at 14° C. to 15° C.

Jones reported his data in survival times of 1 day (14-30 hours), 2 days (38-60 hours), 4 days (76-120 hours), and 1 week (140-200 hours) and in lethal concentration limits, or the concentration of the metals at which the average survival time nearly equaled that of the controls, about 10 days.

For trivalent chromium in the form of $Cr_2(SO_4)_3$ Jones found the following concentrations in relation to survival times: 1 day, 5.0 mg./l.; 2 days, 2.0 mg./l.; 4 days, 1.4 mg./l.; and 1 week, 1.3

mg./l. with a final lethal concentration limit of 1.2 mg./l. or 2.30 x 10^{-5} gram atoms/liter.

Grindley (1945), in his paper on disposal of chromate wastes, reported of tests made on chromium using aerated, distilled water as a diluent. He found rainbow trout, Salmo gairdneri var. shasta, would tolerate a potassium chromate and dichromate solution at concentration equivalent to 20 mg. Cr/l., for from 2 to 8 days. He concluded that the limiting concentration below which substantially neutral solutions of chromate are nontoxic to rainbow trout during periods of 8 days, under experimental conditions, appeared to be slightly less than 20 mg. Cr/l.

In Grindley's (1946) paper on toxicity to fish he again reports on chromate toxicity to rainbow trout. Whether these two papers cover the same experiment could not be determined, since no reference was made in either work to the other project. Assuming two tests were performed, he again used well-aerated distilled water to make up the potassium chromate and dichromate solutions which were maintained at 18° C. during exposure to the fish.

The toxicity of a solution to individual fish was expressed as 100 times the reciprocal of the time period, in minutes, during which each fish was immersed. The average of these individual toxicities was reported as the mean toxicity plus or minus the standard deviation.

Rather than reporting his complete data here, which covered twenty-two dilutions of both K_2CrO_4 and $K_2Cr_2O_7$, only the results which are closest to the 24 hours (1440 minutes) and 48 hours (2880 minutes) lethal concentrations are discussed. With K_2CrO_4 Grindley found 10 trout would endure 200 mg. Cr/l. (747 mg. K_2CrO_4/l.) at an initial pH of 7.3 for an average of 374 minutes before overturning, with a calculated mean toxicity of 0.27 ± 0.11 $min.^{-1}$; 50 mg. Cr/l. (187 K_2CrO_4 mg./l.) at pH 6.6 could be endured 3580 minutes with a mean toxicity of 0.028 ± 0.010 $min.^{-1}$.

With the dichromate his results were as follows: 200 mg. Cr/l. (566 mg. $K_2Cr_2O_7$/l.) at pH 5.4 for 188 minutes with a mean toxocity of 0.53 ± 0.60 $min.^{-1}$, 50 mg. Cr/l. (142 mg. $K_2Cr_2O_7$/l.) at pH 5.0 for 1946 minutes with a mean toxicity of 0.051 ± 0.044 $min.^{-1}$ and 20 mg. Cr/l (57 mg. $K_2Cr_2O_7$/l.) at pH 5.5 for 4348 minutes with a mean toxicity of 0.023 ± 0.008 $min.^{-1}$.

It is interesting to note that the chromate and dichromate, when expressed as mg. Cr/l., yield approximately the same toxicities.

When the fish had turned over, they were transferred to fresh water, and fish which had been immersed in solutions containing the highest concentration of chromate died soon after transfer. Some of the fish, which had been immersed in lower concentrations, died in about 3 days after transfer, and a few did recover. Very

few fish which had been immersed in solutions of the dichromate survived when transferred to fresh water. Those that did survive were usually the fish which had been the first to overturn, suggesting that these fish were affected by the acidity of the solution rather than by any specific toxic effect of the dichromate.

Fromm and Schiffman (1958), using the methods of Doudoroff et al. (1951), found the 48 hour mean tolerance limits of largemouth bass, length 10.6 ± 0.64 cm., weight 11.3 ± 2.7 grams, for hexavalent chromium, K_2CrO_4, was 195 mg. Cr/l. The diluent had a total hardness of 334 mg. $CaCO_3$/l., total alkalinity of 206 mg. $CaCO_3$/l., a pH of 8.5 to 8.5, and was held at 20° C. to 21° C.

In addition to the above, Doudoroff and Katz (1953), in their review of literature pertaining to the toxicity of wastes to fish, cite other references, which, due to their unavailability, the author was not able to consult. They are as follows: Oshima (1931) reported that the survival time of young eels in a solution of $KCr_2(SO_4)_2$ at a concentration of 5.2 mg. Cr/l. averaged 18.7 hours. He also found that 0.01 M. K_2CrO_4 and 0.005 M. $K_2Cr_2O_7$ solutions (520 mg. Cr/l.) were lethal to young eels in an average of 12.4 and 5.35 hours respectively, whereas 0.0025 M. K_2CrO_4 and 0.0005 M. $K_2Cr_2O_7$ solutions (130 mg. Cr/l. and 52 mg. Cr/l.) killed no eels in 50 hours.

Le Clerc and Devlaminck (1950) found that trivalent chromium, as a sulfate, at a concentration of 40 mg. Cr/l. was a minimum fatal concentration for minnows in 6 hours. Rushton (1921) found that a 6 hour exposure to 100 mg. $K_2Cr_2O_7$/l. proved fatal to trout within 12 hours after return to fresh water.

Klassen et al. (1949) did work on wastes from a chromium plating industry in Woodstock, Illinois. Since the author is personally familiar with this work, he feels that the results were so erratic that they do not constitute a valid test. Doudoroff, in his report on this work, cited that in one test 10 bluegill sunfish tolerated 83 mg. Cr/l. for 10 days or more, whereas 6 bluegill sunfish died within 4 days in a diluted waste containing 75 mg. Cr/l. It is the author's opinion that these conflicting data were due to the fact that the waste used in the bioassays was collected at various time periods and its composition was in a state of constant flux. Knowing the situation, cyanide would be suspected as being a contributing factor to the variation observed.

The data relating to the toxicity of chromium to invertebrates is even more scarce than that of the fish.

Anderson has made excellent progress in the field with his work with Daphnia magna in an attempt to study the effect of various pollutants on the food chain of fish. In his work (1944, 1946) he

used threshold concentrations; that is, concentrations which would immobilize 50 percent of the test animals in a given period of time. In his earlier work, he employed a time interval of 16 hours, but found this unsatisfactory. Then he changed to a 48 hour period in 1946 with a resultant lowering of thresholds. In both these projects, he used Lake Erie water as the diluent which had the following pertinent chemical composition: calcium, 31 mg./l.; magnesium, 7.6 mg./l.; carbonate radical, 3.1 mg./l.; bicarbonate radical, 114 mg./l.; sulfate radical, 13 mg./l.; chlorine (Cl), 8.7 mg./l.; and total dissolved solid, 133 mg./l.

In 1944 Anderson found K_2CrO_4 to immobilized Daphnia magna in 16 hours at a concentration of less than 0.6 mg./l. In his 1946 work with sodium salts, he found little difference between the chromate and dichromate thresholds when compared on the basis of the chromium content of the molecule, the chromate being toxic at <0.32 mg./l. and the dichromate at $<<0.31$ mg./l.

Rudolfs et al. (1950) reported a toxic effect to flora and fauna due to trivalent chromium concentrations of 6 mg./l. or less. His article, a comprehensive review of toxicity literature pertaining to all branches of waste disposal, was lengthy, and reference to the particular work in which this datum was found could not be located.

APPENDIX II

This section contains all data and their statistical evaluation. All statistical methods, except the analysis of variance technique used on the regression coefficients, are according to Snedecor (1956). His convenient abbreviations, * meaning significant (α = .05) and ** meaning highly significant (α = .01) are used.

The analysis of variance techniques used on the regression coefficients are those of Dr. W. D. Baten, Statistician, Michigan State University Agriculture Experiment Station.

Part A. Normal Trout

Data Used to Obtain the Regression Curves of Kidney, Liver, and Spleen Weights on Body Weight

Total Wt. of Fish Minus Organs (g.)	Total Length of Fish (cm.)	Kidney Weight (mg.)	Kidney Index (pct. body wt.)	Liver Weight (mg.)	Liver Index (pct. body wt.)	Spleen Weight (mg.)	Spleen Index (pct. body wt.)
14.6	13.1	131.8	0.90	66.6	0.46	32.6	0.22
11.0	11.3	79.8	0.72	130.0	1.18	36.2	0.33
8.7	11.6	64.6	0.74	74.4	0.86	13.4	0.15
14.8	12.9	81.6	0.55	162.0	1.09	46.2	0.31
17.7	13.2	176.0	0.99	181.0	1.02	54.0	0.30
13.7	12.7	120.4	0.88	170.4	1.24	27.4	0.20
24.2	14.6	199.6	0.82	231.8	0.96	79.2	0.33
14.0	12.5	93.6	0.67	153.4	1.10	27.4	0.20
11.1	12.5	82.8	0.74	103.8	0.94	15.6	0.14
26.0	15.6	200.0	0.77	345.4	1.33	51.4	0.20

Data Pertaining to Organ Weight, Gall Bladder, and Gonads

Total Weight of Fish Minus Organs (g.)	Total Length of Fish (cm.)	Gall Bladder Weight (mg.)	Gall Bladder Index (pct. body wt.)	Gonad Weight (mg.)	Gonad Index (pct. body wt.)
14.6	13.1	49.0	0.34	8.6	0.06
11.0	11.3	20.0	0.18	34.2	0.31
8.7	11.6	25.0	0.29	25.6	0.29
14.8	12.9	36.4	0.25	44.6	0.30
17.7	13.2	18.0	0.10	379.0	2.14
13.7	12.7	24.0	0.18	6.4	0.05
24.2	14.6	31.8	0.13	272.0	1.12
14.0	12.5	27.0	0.19	34.8	0.24
11.1	12.5	24.6	0.22	11.6	0.10
26.0	15.6	38.6	0.15	28.0	0.11

Data Used to Obtain Regression Curves of Total Water Weight on Body Weight

Total Weight of Fish Minus Organs (g.)	Total Length of Fish (cm.)	Dish Weight (g.)	Dish Wt. Plus Dry Fish Wt. (g.)	Water Weight (g.)	Water Weight (pct. body wt.)
14.6	13.1	59.3	62.6	11.3	77
11.0	11.3	59.8	62.5	8.3	75
8.7	11.6	60.5	62.5	6.2	71
14.8	12.9	58.2	61.5	11.5	78
17.7	13.2	65.4	69.6	13.5	76
13.7	12.7	57.3	60.2	10.8	79
24.2	14.6	64.4	69.9	18.7	77
14.0	12.5	65.1	68.3	10.8	77
11.1	12.5	58.8	60.8	9.1	82
26.0	15.6	54.8	59.5	21.3	82

Data Pertaining to Hematocrit, Red Blood Cell Count, Hemoglobins, and Red Blood Cell Length

Total Weight of Fish (g.)	Total Length of Fish (cm.)	Hematocrit (pct. RBC)	Red Blood Cell Count (millions/ cmm.)	Hemoglobin (g. pct.)	Cell Length $\bar{X}$ (μ.)
15.5	13.5	35	1.08	5.3	14.6
16.1	12.6	35	1.12	8.3	14.7
21.4	14.0	30	1.15	7.4	15.2
12.2	11.7	39	1.04	8.1	14.6
23.0	14.0	26	0.99	6.3	14.4
20.8	15.2	27	1.19	5.3	14.7
11.8	11.3	28	1.26	7.2	14.6
11.7	11.4	36	1.08	6.3	15.0
18.7	13.3	29	1.22	6.5	14.7
21.7	14.1	33	0.96	4.4	14.4

Data Used to Obtain the Regression Curves of Plasma and Blood Volumes on Body Weight

Total Weight of Fish (g.)	T-1824 Injected (mg.)	T-1824 Recovered (mg./ml.)	Hematocrit (pct. RBC)	Plasma Volume (ml.)	Plasma Pct. of Total Weight	Blood Volume (ml.)	Blood Pct. of Total Weight
14.5	0.147	0.377	29	0.39	2.7	0.54	3.8
29.1	0.147	0.273	34	0.54	1.9	0.82	2.8
22.2	0.074	0.198	38	0.37	1.7	0.60	2.7
14.4	0.074	0.393	47	0.19	1.3	0.36	2.5
22.2	0.074	0.155	37	0.48	2.2	0.76	3.4
14.6	0.074	0.178	27	0.42	2.8	0.57	3.9
10.5	0.074	0.315	34	0.23	2.2	0.36	3.4
20.7	0.074	0.178	28	0.42	2.0	0.58	2.8
12.2	0.074	0.299	40	0.25	2.0	0.41	3.4
13.5	0.074	0.222	36	0.33	2.5	0.52	3.8

Statistical Values of the Data on Normal Controls

Value	Kidney Weight	Liver Weight	Spleen Weight	Total Body Water
Mean ($\bar{X}$)	0.77 pct.	1.02 pct.	0.24 pct.	77.4 pct.
Variance (s^2) . . .	0.016	0.059	0.005	10.49
Standard Deviation (s) . . .	0.013	0.24	0.08	3.24
Standard Error ($s_{\bar{X}}$)	0.039	0.077	0.023	1.02

Statistical Values of the Data on Normal Controls (Continued)

Hematocrit	Hemoglobin	RBC Count	RBC Length	Plasma Volume	Blood Volume
31.8 pct.	6.51 g./ 100 ml.	1.11×10^6 per cmm.	14.67 μ.	2.13 pct.	3.25 pct.
19.33	1.61	0.0096	0.84	0.188	0.26
4.39	1.27	0.098	0.92	0.42	0.51
1.39	0.40	0.03	0.092	0.14	0.16

Statistical Values Related to the Regression of the Above on Body Weight

Value	Kidney Weight	Liver Weight	Spleen Weight	Total Body Water	Plasma Volume	Blood Volume
Reg. Coef. (b)	2.97	12.89	8.57	0.82	0.015	0.023
Intersect (a)	-7.93	-38.95	-10.50	-0.62	0.094	0.05
Correl. Coef. (r)	0.84	0.88	0.93	0.996	0.80	0.89
Standard Error of Estimate	11.53	40.46	20.43	0.47	0.073	0.077

Regression analysis

Kidney weight.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	24,160.92	
Regression	1	20,822.87	20,822.87**
Error	8	3,338.05	417.26

Analysis of the correlation coefficient:

$t = r\sqrt{(m-2)} / 1-r^2$, d.f. = n-2.

t = 19.39**

Liver weight.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	60,199.14	
Regression	1	47,103.41	47,103.41**
Error	8	13,095.73	1,636.96

Analysis of the correlation coefficient:

t = 11.00**

Spleen weight.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	3563.12	
Regression	1	2501.07	2501.07**
Error	8	1062.05	132.76

Analysis of the correlation coefficient:
t = 8.05**

Total body water.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	192.77	
Regression	1	190.99	190.99**
Error	8	1.78	0.22

Analysis of the correlation coefficient:
t = 140.29**

Plasma volume.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	0.1138	
Regression	1	0.0715	0.0715**
Error	8	0.0423	0.0053

Analysis of the correlation coefficient:
t = 6.27**

Blood volume.

Analysis of Variance of Regression Coefficient

Source	Degrees of Freedom	Sum of Squares	Mean Square
Total	9	0.2136	
Regression	1	0.1657	0.1657**
Error	8	0.0479	0.0060

Analysis of the correlation coefficient:
t = 12.07**

Part B. Chromium-induced Changes

Data Used for the Straight-Line Interpolation of the 24-Hour Median Tolerance Limit

Chromium (mg./l.)	Number of Fish	24-Hour Survival (pct.)
71.0	10	100
97.0	10	70
127.0	10	0
135.0	10	10
144.0	10	0
154.0	10	0

Data Used to Calculate the Activity of the Aquaria Water in Tracer Experiments

Background (count/ 5 min.)	Background (cpm)	Water (count/ 5 min.)	Water (cpm)	Corrected Water (cpm/ml.)
190	38	263	53	15
195	39	252	50	11
184	37	217	46	9
Mean				12

Data Used to Determine Amount of Chromium in Aquaria Water in Tracer Experiment

Sample No.	Pct. Trans.	Chromium (μg./sample)	Chromium (mg./l.)
1	44.0	9.3	18.6
2	43.5	9.3	18.6
3	43.5	9.3	18.6

Weights of the Organs of Fish Used in Tracer Experiment

Fish Wt. (g.)	Spleen Wt. (mg.)	Liver Wt. (mg.)	Gall Bladder Wt. (mg.)	Kidney Wt. (mg.)	Caudal Peduncle Wt. (mg.)
8.1	17.0	131.8	16.2	78.0	235.4
14.7	21.6	158.0	24.8	94.4	170.4
15.8	63.0	174.2	41.0	128.2	218.0

Activity of Organ of Fish Exposed to Radioactive Chromium

Spleen Count (5 min.)	Liver Count (5 min.)	Gall Bladder Count (5 min.)	Kidney Count (5 min.)	Caudal Peduncle Count (5 min.)
182	190	184	204	206
193	234	186	220	179
186	204	183	213	203

Organ Counts Corrected to Counts per Minute per 100 mg. of Organ

Spleen Count Corrected (cpm/ 100 mg.)	Liver Count Corrected (cpm/ 100 mg.)	Gall Bladder Count Corrected (cpm/ 100 mg.)	Kidney Count Corrected (cpm/ 100 mg.)	Caudle Peduncle Count Corrected (cpm/ 100 mg.)
43.53	6.83	48.14	15.30	5.18
44.44	11.26	33.06	15.89	3.99
13.10	6.77	18.54	10.61	5.32

Analysis of Variance of Tracer Data

Source	f	Sums of Squares	Mean Square
Total	14	3386.13	
Between	4	2273.83	568.46**
Error	10	1112.30	111.23

$s_{\bar{X}} = 6.09$

Q Test (Studentized Range)

	2	3	4
Q	3.01	3.67	4.08
$Qs_{\bar{X}}$	22.35	24.84	26.61

Spleen	34.92 - 26.61 = 8.31
Gall bladder	33.24 - 24.84 = 8.40
Kidney	13.88
Liver	8.28
Caudal peduncle	4.83

Organ Weight, Total Body Water, and Hematocrit Data Which Was Compared with Normal Control Data

Fish Wt. (g.)	Water Wt. (g.)	Spleen Wt. (mg.)	Liver Wt. (mg.)	Kidney Wt. (mg.)	Hematocrits (ml./ 100 ml.)
7.2	5.6	10.0	94.0	62.8	56
10.2	8.4	17.2	153.2	68.4	--
15.6	11.7	44.0	178.0	92.4	53
13.8	10.1	18.8	186.0	129.4	53
12.3	9.6	17.4	166.0	99.6	51

Data from Normal Fish Exposed to 18 mg. Cr/l.
(normal chromium group)

Hematocrit (ml./ 100 ml.)	Red Blood Cells (millions/cmm.)	Hemoglobin (g./ 100 ml.)	Cell Length (μ)	Fish Length (cm.)	Fish Weight (g.)
36	1.21	5.5	14.2	14.6	23.2
45	1.19	6.1	15.6	10.0	7.8
43	1.30	7.6	15.2	12.9	12.9
43	1.33	6.9	14.7	14.0	14.6
46	1.12	6.7	14.7	10.9	9.4
42	1.37	6.1	14.1	12.6	15.3
38	1.05	5.6	16.5	16.9	33.9
44	1.26	6.5	--	12.1	12.0
54	1.28	7.2	15.0	10.7	8.5
47	1.39	7.8	14.4	13.2	17.4

Plasma and Blood Volume Data from Normal Fish Exposed to 18 mg. Cr/l. (normal chromium group)

Plasma Vol. (ml./ 100 g.)	Wt. (g.)	Length (cm.)	T-1824 Injected (mg.)	T-1824 Recovered (mg./ ml.)	Hematocrit (ml./ 100 ml.)	Plasma Vol. (ml.)	Blood Vol. (ml.)	Blood Vol. (ml./ 100 g. body wt.)
1.3	26.2	14.9	0.074	0.221	58	.33	.80	3.0
2.3	12.7	12.4	0.074	0.257	45	.29	.52	4.1
2.2	38.6	17.0	0.074	0.086	35	.86	1.32	3.4
1.1	33.0	15.9	0.074	0.198	60	.37	.94	2.8
1.8	14.7	13.0	0.074	0.280	51	.26	.54	3.7
1.5	20.9	14.9	0.074	0.236	48	.31	.60	2.9
1.4	31.4	16.3	0.074	0.164	49	.45	.88	2.8
1.5	34.0	16.4	0.074	0.147	40	.50	.84	2.5
1.5	14.0	13.0	0.074	0.343	34	.22	.33	2.3
1.5	21.2	14.6	0.074	0.239	43	.31	.55	2.6

Data from Splenectomized Fish Exposed to Tap Water (splenectomized control)

Length (cm.)	Weight (g.)	Hematocrit (ml./100 ml.)	Red Blood Cell Count (millions/ cmm.)	Hemoglobin (g./100 ml.)
11.8	12.9	28	1.08	6.3
12.3	11.9	23	.99	5.6
10.6	7.5	27	1.04	4.7
13.9	15.3	24	.68	4.0
13.7	16.7	26	1.18	5.4
14.4	16.5	30	.85	5.1
13.1	17.8	34	1.22	7.2
13.3	17.3	25	.92	4.4
14.1	18.3	37	1.34	7.8
13.4	19.0	31	1.10	5.6

Plasma and Blood Volume Data from Splenectomized Fish Exposed to Tap Water (splenectomized control group)

Length (cm.)	Weight (g.)	T-1824 Injected (mg.)	T-1824 Recovered (mg./ml.)	Plasma Vol. (ml.)	Plasma Vol. (ml./100 g.)	Hematocrit (ml./100 ml.)	Blood Vol. (ml.)	Blood Vol. (ml./100 g.)
15.8	34.6	0.074	0.093	.80	2.3	47	1.50	4.3
16.0	31.6	0.074	0.103	.72	2.3	33	1.07	3.4
15.2	22.4	0.074	0.105	.70	3.1	32	1.04	4.6
16.0	34.0	0.074	0.110	.67	2.0	40	1.12	3.3
15.2	26.5	0.074	0.147	.50	1.9	44	.90	3.4
14.0	19.5	0.074	0.134	.55	2.8	27	.76	3.9
12.4	14.6	0.074	0.215	.34	2.4	36	.54	3.7
12.7	13.5	0.074	0.192	.38	2.8	37	.61	4.5
14.2	21.4	0.074	0.147	.50	2.4	44	.90	4.2
15.9	26.3	0.074	0.118	.63	2.4	26	.85	3.2

Data from Splenectomized Fish Exposed to 24.8 mg. Cr/l. (splenectomized chromium group)

Length (cm.)	Weight (g.)	Hematocrit (ml./100 ml.)	Red Blood Cell Count (millions/ cmm.)	Hemoglobin (g./100 ml.)
13.7	20.6	53	1.57	9.4
13.8	15.6	40	1.31	8.1
12.9	11.9	52	1.97	8.1
13.8	16.2	42	1.50	8.1
13.2	15.2	25	0.98	4.9
15.1	21.3	32	0.92	5.4
16.7	35.1	47	1.74	8.3
14.8	18.6	41	1.35	8.3
15.3	21.3	30	0.99	5.1
13.0	13.9	44	1.43	8.5

Plasma and Blood Volume Data from Splenectomized Fish Exposed to 24.8 mg. Cr/l.

Length (cm.)	Weight (g.)	T-1824 Injected (mg.)	T-1824 Recovered (mg./ml.)	Plasma Vol. (ml.)	Plasma Vol. (ml./100 g.)	Hematocrit (ml./100 ml.)	Blood Vol. (ml.)	Blood Vol. (ml./100 g.)
15.8	33.3	0.074	0.116	.64	1.9	36	1.00	3.0
13.9	17.4	0.074	0.247	.30	1.7	53	.64	3.6
15.6	29.5	0.074	0.196	.38	1.3	48	.73	2.5
13.9	18.2	0.074	0.230	.32	1.8	50	.64	3.5
12.9	15.3	0.074	0.350	.21	1.4	52	.44	2.9
14.0	18.8	0.074	0.264	.28	1.5	46	.52	2.8
13.8	15.7	0.074	0.221	.33	2.1	35	.51	3.3
13.9	14.8	0.074	0.204	.36	2.4	39	.59	4.0
14.6	21.4	0.074	0.210	.35	1.6	47	.67	3.1
14.7	19.8	0.074	0.221	.33	1.7	43	.58	2.9

Regression of plasma volume on body weight

Regression of Plasma Volume on Body Weight

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Regression coefficient (b)	0.016	0.017	0.015
Intersect (a)	0.00	0.016	0.05
Correlation coefficient (r)	0.81	0.86	0.81
Standard error of est. ($S_{\bar{e}}$)	0.116	0.084	0.069
"t" value of correlation coefficient .	6.64**	9.31**	6.67**

Analysis of variance tables--testing regression coefficients.

Normal Chromium

Source	f	Sums of Squares	Mean Square
Total	9	0.3092	
Regression	1	0.2016	0.2016**
Error	8	0.1076	0.0135

Splenectomized Control

Source	f	Sums of Squares	Mean Square
Total	9	0.2042	
Regression	1	0.1483	0.1483**
Error	8	0.0559	0.0070

Splenectomized Chromium

Source	f	Sums of Squares	Mean Square
Total	9	0.1138	
Regression	1	0.0763	0.0763**
Error	8	0.0375	0.0047

Regression of blood volume on body weight

Regression of Blood Volume on Body Weight

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Regression coefficient (b)	0.028	0.032	0.022
Intersect (a)	0.04	0.14	0.18
Correlation Coefficient (r)	0.92	0.88	0.88
Standard error of est. ($S_{\bar{e}}$)	0.116	0.14	0.077
"t" value of correlation coefficient .	16.89**	11.00**	11.00**

Analysis of variance table--testing regression coefficients.

Normal Chromium

Source	f	Sums of Squares	Mean Square
Total	9	0.2136	
Regression	1	0.1657	0.1657**
Error	8	0.0479	0.0060

Splenectomized Control

Source	f	Sums of Squares	Mean Square
Total	9	0.2042	
Regression	1	0.1483	0.1483**
Error	8	0.0559	0.0070

Splenectomized Chromium

Source	f	Sums of Squares	Mean Square
Total	9	0.2154	
Regression	1	0.1679	0.1679**
Error	8	0.0475	0.0059

Statistical values for hematocrits

Statistical Values for Hematocrits

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Mean ($\bar{X}$)	43.8	28.5	44.9
Variance (s^2)	24.44	20.33	41.44
Standard deviation (s)	4.94	4.50	6.44
Standard error ($s_{\bar{X}}$) .	1.56	1.42	1.36

Statistical values for red blood cell counts

Statistical Values for Red Blood Cell Counts

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Mean ($\bar{X}$)	1.25	1.04	1.38
Variance (s^2)	0.01	0.33	0.12
Standard deviation (s)	0.10	0.57	0.34
Standard error ($s_{\bar{X}}$) .	0.032	0.18	0.11

Statistical values for hemoglobin

Statistical Values for Hemoglobin

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Mean ($\bar{X}$)	6.60	5.16	7.42
Variance (s^2)	0.62	12.99	2.65
Standard deviation (s)	0.79	3.60	1.62
Standard error ($s_{\bar{X}}$) .	0.25	1.14	0.51

Statistical values for plasma volume as percent of total body weight

Statistical Values for Plasma Volume as Percent of Total Body Weight

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Mean ($\bar{X}$)	1.61	2.44	1.74
Variance (s^2)	0.15	0.14	0.108
Standard deviation (s)	0.38	0.37	0.33
Standard error ($s_{\bar{X}}$) .	0.12	0.12	0.10

Statistical values for blood volume as percent of total body weight

Statistical Values for Blood Volume as Percent of Total Body Weight

	Normal Chromium	Splenectomized Control	Splenectomized Chromium
Mean ($\bar{X}$)	3.01	3.85	3.16
Variance (s^2)	0.32	0.27	0.20
Standard deviation (s)	0.56	0.52	0.44
Standard error ($s_{\bar{X}}$) .	0.18	0.16	0.14

Covariance analysis of plasma volume data

Covariance Analysis of Plasma Volume Data

Source	f	Σd^2	Mean Square
Normal control	8	0.040	0.005
Splenectomized control	8	0.054	0.007
Normal chromium	8	0.106	0.013
Splenectomized chromium	8	0.039	0.005
Within	32	0.238	0.007
Regression coefficient	3	0.003	0.001
Common (error)	35	0.241	0.007
Adjusted means	3	0.218	0.073**
Total	38	0.459	

$S_{\overline{yA}} = 0.026$

"Q"-Test

	2	3	4
Q	2.87	3.46	3.81
QSyA	0.075	0.090	0.099

Splenectomized chromium	.579 - .099 = .480
Normal chromium	.390 - .090 = .300
Normal control	.362 - .075 = .287
Splenectomized control	.350

Covariance analysis of blood volume data

Covariance Analysis of Blood Volume Data

Source	f	Σd^2	Mean Square
Normal control	8	0.046	0.006
Splenectomized control	8	0.136	0.017
Normal chromium	8	0.107	0.013
Splenectomized chromium	8	0.140	0.018
Within	32	0.429	0.013
Regression coefficient	3	0.35	0.012
Common (error)	35	0.464	0.013
Adjusted means	3	0.115	0.038*
Total	38	0.579	

$S_{\overline{yA}} = 0.079$

"Q"-Test

	2	3	4
Q	2.87	3.46	3.81
QSyA	.227	.273	.301

Splenectomized control	.924 - .301 = .628
Normal chromium	.732 - .273 = .459
Splenectomized chromium	.632 - .227 = .405
Normal control	.552

Analaysis of variance of hematocrit data

Analysis of Variance of Hematocrit Data

Source	f	Sums of Squares	Mean Square
Total	39	2902	
Between	3	1557	519**
Error	6	1345	37.3

$S_{\bar{X}} = 1.93$

"Q"-Test

	2	3	4
Q	2.87	3.46	3.82
$QS_{\bar{X}}$	5.64	6.68	7.37

Normal chromium	43.8 - 7.37 = 36.4
Splenectomized chromium	40.6 - 6.68 = 33.9
Normal control	31.8 - 5.64 = 26.2
Splenectomized control	28.5

Analysis of variance of hemoglobin data

Analysis of Variance of Hemoglobin Data

Source	f	Sums of Squares	Mean Square
Total	39	73.35	
Between	3	16.43	5.48*
Error	36	56.92	1.55

$S_{\bar{X}} = 0.39$

"Q"-Test

	2	3	4
Q	2.87	3.46	3.82
$QS_{\bar{X}}$	1.12	1.35	1.49

Splenectomized chromium	7.42 - 1.49 = 5.93
Normal chromium	6.60 - 1.35 = 5.25
Normal control	6.51 - 1.12 = 5.39
Splenectomized control	5.61

Analysis of variance of red blood cell count data

Analysis of Variance of Red Blood Cell Count Data

Source	f	Sums of Squares	Mean Square
Total	39	2.26	
Between	3	0.67	0.22**
Error	36	1.59	0.044

$S_{\bar{X}} = 0.066$

"Q"-Test

	2	3	4
Q	2.87	3.46	3.82
$QS_{\bar{X}}$	0.19	0.21	0.25

Splenectomized chromium	1.38 - 0.25 = 1.13
Normal chromium	1.25 - 0.21 = 1.04
Normal control	1.11 - 0.19 = 0.92
Splenectomized control	1.04

"t"-Tests

Between normal chromium and normal control:

Red blood cell counts $t = 3.18^{**}$

Red blood cell lengths $t = 4.77^{**}$

Data used in potassium chloride experiments--hematocrits

Data Used in Potassium Chloride Experiments--Hematocrits

	Tap Water	29.8 mg. K/l.	20 mg. Cr/l.
Samples	39	38	47
	32	44	44
	33	37	33
	30	38	44
	34	45	40
	36	34	35
	28	35	43
	32	39	34
	37	35	38
	39	43	44
Mean	34.0	38.8	40.2
Variance	13.77	85.55	24.44
Standard deviation . .	3.71	9.25	4.9
Standard error	1.17	2.92	1.56

"t"-Tests.

Between hematocrits from KCl solution and tap water:

$t = 1.53$

Between hematocrits from KCl and Cr solutions:

$t = 0.42$

Data used in determination of spleen function

Results of Hematocrits Determined on Sham Operated and Splenectomized Fish

Splenectomized Control No T-1824 $Time_{10}$		Sham Operated $Time_0$	Sham Operated $Time_{10}$
26	36	29	30
32	29	38	35
38	32	33	40
36	50	32	36
44	26	36	38