ALLOXAN DIABETES

IN THE

CATFISH

ALLOXAN DIABETES IN THE CATFISH

By

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SCOPE AND CONTENTS: Healthy adult catfish were injected with 10% aqueous alloxan (400mg/kg) in the conus arteriosus. Blood glucose, determined by a modified Folin-Malmros micro technique, showed hyperglycemia persistent from 3 hours to 14 days for alloxanized animals, but no significant change for distilled-water-injected fish.

Pancreatic islets from alloxanized and control fish sacrificed at representative stages were examined by a modified Gomori aldehyde-fuchsin technique. Beta cell degranualtion and nuclear changes, roughly parallel to glucose response, were apparent in alloxan treated animals.

This preliminary study suggests that future work should include (1) attempts to induce diabetes by other methods, (2) additional study of pancreatic histology, and (3) extension of histological studies to other organs.

PREFACE

This investigation represents one stage in a phyletic survey of experimental diabetes in the lower vertebrates being conducted by Dr. P. F. Nace. Previous reports have dealt with the toadfish, the chicken and the hamster.

The present work has been supported by grants from The National Research Council of Canada, and The National Cancer Institute of Canada, to whom thanks are extended. The method for determination of blood glucose was devised during a study of carbohydrate metabolism in the toadfish, supported by The National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, Grant Number A1129Endo, to Dr. P. F. Nace and The Marine Biological Laboratory, Woods Hole, Massachusetts.

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INTRODUCTION

Human diabetes has been recognized in many parts of the world since ancient times (52). It represents one of the few conditions whose cause has been traced first to an organ, to a particular region in that organ, then to a cell type in that region, and, finally, to a cytoplasmic granule in that cell (25). In spite of this presumably complete knowledge, there is, at present, neither an adequate definition of "diabetes", nor any precise idea of the relationships between clinical and experimental diabetes (1). All that can be said is that diabetes is a disturbance in the complex interplay among the adrenal cortex, anterior hypophysis, thyroid and endocrine pancreas as primary centers, involving many other parts of the organism, and which manifests itself in a profound upset of carbohydrate metabolism (38). Most diabetes is marked by degranulation of the pancreatic beta cells, but varieties are known, both in man and animals, in which no structural abnormality has been detected (38) in the pancreas.

Diabetes was first produced experimentally by von Mering and Minkowski in 1889 (45), who performed partial pancreatectomies in dogs. Since that time, it has been produced in a variety

of fashions in several species. Woerner (68) found that large intravenous doses of glucose were diabetogenic, and other workers have established the condition by injections of the cortical steroids. (34, 27), anterior pituitary extracts (26, 31, 33) and by alloxan (11).

Alloxan (pyrimidinetetrone) was first noticed to produce hypoglycemia in rabbits by Jacobs in 1937 (36). He presumably did no further work with the compound, and the first complete recognition of alloxan diabetes was the observation by Dunn, Sheehan and McLetchie in 1943 (11) of hyperglycemia and islet damage in the rabbit following intravenous alloxan. Since that time, alloxan has been administered to a number of species including rats (43), dogs (43), hamsters (30), sheep (13), monkeys (2, 46), frogs (62), toads (32), turtles (17, 42), man (7), several species of birds (18, 47, 59) and a few species of fish (23, 58, 51, 39, 48).

Until recently, however, most of the investigations in the area have centered about the common laboratory mammals. Work on these forms has contributed much to the understanding of diabetes, but has failed to provide the answers to a number of basic questions. The lack of agreement on the nature of some basic features of diabetes has been pointed out by the recent report (March, 1958) of Lazarus and Volk (40) on the mechanism of tolbutamide action in alleviating hyperglycemia. A problem of particular interest is presented by current ignorance of factors concerned

in recovery from diabetes by regeneration of damaged or destroyed beta cells. For attack on this problem, several characteristics of poikilotherms appear significant. Particularly worthy of mention are their slow metabolic rates, their regenerative capacities (22) and their larger cells. The same animal groups may be valuable for other problems of diabetes. Comparative physiology has provided understanding of many mammalian functions, perhaps most notably in the work of Smith (64) on the kidney of Opsanus.

Among the poikilotherms to which alloxan has been administered, there has been a wide range of response. The frog develops islet changes, but no hyperglycemia (62). The toad shows no changes (32), but the reason for this is unknown. The earliest report on the turtle indicates no islet lesions and only a transient hyperglycemia (17), though a later report by another author (42) presents evidence for hydropic degeneration of islets in a few individuals 52 - 60 days after alloxan.

Among poikilotherms, fish enjoy particular advantages for work of this type. They are readily available in large numbers and have convenient anatomical features for intravascular injection and blood sampling, and for histological examination.

Despite these features, little attention has been directed to experimental diabetes in this class. Saviano (58) has reported alloxan diabetes in a selacian fish, Seyllium canicula, which develops

hyperglycemia and islet damage. Doerr (10) and Pallot and Schatzle (51) have alloxanized cyprinoids but disagree on details of response. Guppies have been kept in dilute alloxan solutions for several hours (23), with pancreatic and other changes reported as the result of the treatment. Interpretation of this work is modified by the report of Patterson, Lazarow and Levy (53), which demonstrates that alloxan decomposes rapidly in aqueous solutions at 37° C. and pH. 7.4. This, accompanied by reports (5, 41) showing that alloxan cannot be detected in the blood 15 minutes after injection, suggests that the changes observed in the guppy may not be related directly to alloxan administration. The fish which has received the most complete treatment in this area has been the toadfish. Reports by Lazarow (39) and Nace (48,49) are indicative of the general trend of the studies, but there are still many obscure points. Hyperglycemia and islet damage have both been reported in this animal, but they do not always occur together in a given individual (49). The widely divergent results in various reports on the same species make it clear that future research must utilize the disciplines of biochemistry and histology simultaneously. It may be anticipated that such work may clarify some of the unsolved problems of diabetes, such as the mechanism of its origin, the function (both normal and diabetic), of pancreatic alpha cells, the mechanism of recovery from

diabetes, and, possibly, some idea concerning the general features controlling regeneration.

There is increasing evidence that there is some as yet unknown relationship between cardiovascular disease and diabetes. More diabetics die as a direct result of cardiovascular disease than do members of any other group (1). Ingle (35), Goranson and Tilser (21) and others (6) have demonstrated that the rate of tumor growth can be altered by diabetes in experimental animals. This effect may be vascular in origin, or have an entirely different mechanism. A more complete understanding of the precise relationships among these conditions must await a better knowledge of each of the separate areas, as well as integrated studies on combinations of the conditions undertaken from the dual viewpoints of biochemistry and histology.

With these problems in mind, the present work was launched with the following objectives:

(1) Examination of the suitability of the catfish for the study of alloxan diabetes, as evaluated in terms of gross anatomy, histology and blood chemistry;

(2) Description of the normal histology of the catfish endocrine pancreas;

(3) Determination of the animal's normal blood glucose range;

(4) Investigation of the blood glucose response of the catfish to intravascular alloxan;

(5) Evaluation of the histological changes of the endocrine pancreas following alloxan treatment.

MATERIALS AND METHODS

Choice of Animal

Among the considerations important in selecting a fresh water teleost for these studies are hardiness, general resistance to short periods out of water, and anatomical suitability to intravascular injections and repeated blood samplings. Secondary considerations include the presence of reasonably large and discrete islets of Langerhans, and ready availability in large numbers.

The common lake catfish fits all these requirements ideally. In 1926 Simpson (63) reported that periods of anoxia of greater than 20 minutes were needed to produce hyperglycemia in Ameiurus. Our own experience has shown that animals survive well after being out of water for as long as 2 hours. In the treatment or blood sampling in the present study, the fish were out of the water for less than 5 minutes. It has also been shown (63)that blood may be removed repeatedly from the conus arteriosus of this species without permanent injury.

McCormick (44), Diamare (8), Rennie (55) and Vincent and Thompson (67) have suggested that the anatomy and pancreatic histology of the catfish are suitable for these studies. These reports

appeared many years before the development of specific pancreatic beta cell staining procedures (3, 20, 56). The inability of the previous investigators to identify the cell types now considered important in the islet made a re-examination of the normal histology an essential step in the present work.

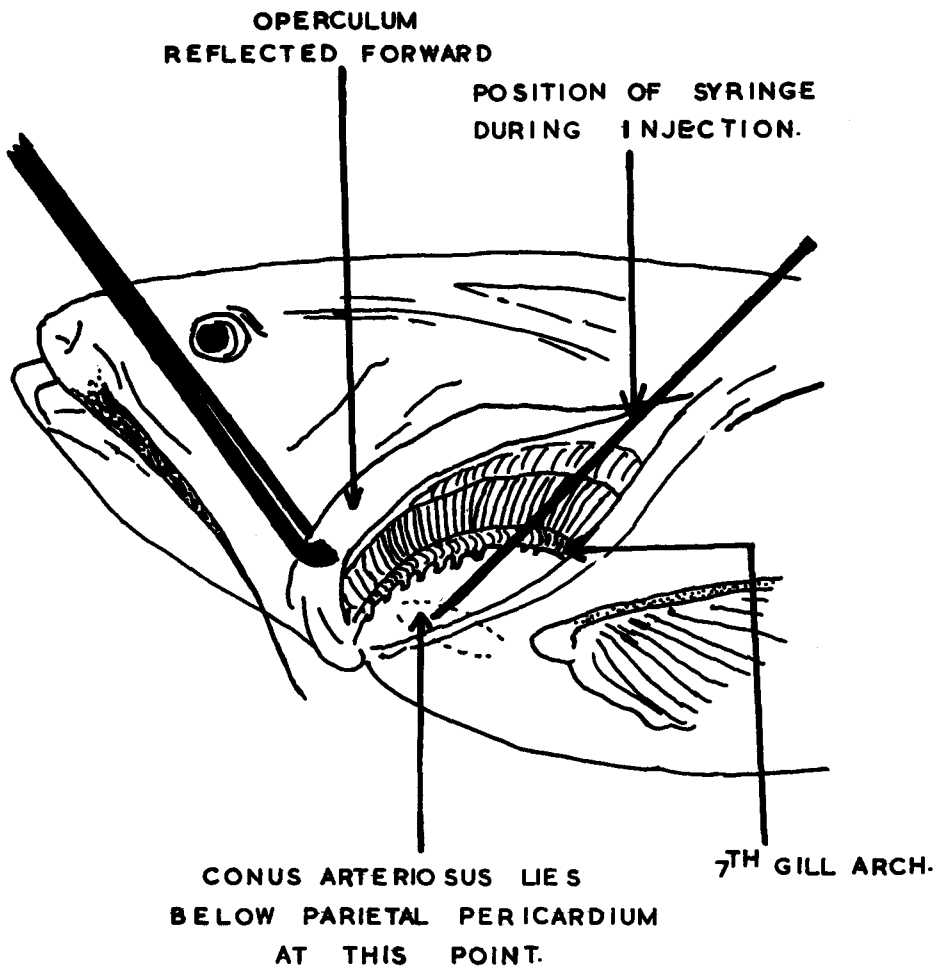
Design of Experiment

Healthy, adult catfish[1] ranging from 200 to 600 grams, weighed to the nearest 5 grams and identified by plastic tags through the dorsal fin, were placed in groups of 10 in aquaria provided with air bubblers and running, charcoal filtered tap water at 10° - 15° C. In each group, 5 fish were injected with iced 10% alloxan (Eastman, Lot #42) in distilled water, administered within 5 minutes of solution. The remaining 5 animals were injected with iced distilled water, and served as controls both for blood glucose and histological analyses. Blood samples were withdrawn from all animals immediately before injection and at regular intervals from 3 hours to 14 days later.

All injections and blood samplings were carried out through the conus arteriosus (Figure 1) as suggested by Simpson (63). The blood glucose was analysed by a modified Folin - Malmros micro

[1] Ictalurus nebulosus (61), formerly Ameiurus nebulosus. Obtained from Lake Erie through the Port Rowan Fishermen's Co- Op.

FIGURE 1. APPROACH TO CONUS ARTERIOSUS FOR INTRAVASCULAR INJECTION.



technique, as outlined in the appendix.

At representative stages from 3 to 96 hours after injection, all surviving animals in 9 groups of 10 individuals each were sacrificed by concussion. The pancreatic islets were examined histologically and degree of beta granulation, as shown by a Gomori aldehyde-fuchsin stain (20) was estimated.

Three additional groups of fish were used: 2 for determination of a diabetogenic dose level of alloxan; 1 for preliminary investigation of blood glucose response to alloxan for periods up to 14 days. No histological studies were done on this latter group.

A few deaths at various stages are responsible for the deviations from the expected number of glucose analyses and histological investigations. No histological work was attempted on animals found dead, because of the rapid post mortem changes known to occur in islet tissue.

In all 416 blood glucose analyses were conducted: 237 on untreated or distilled water injected fish and 179 on alloxanized animals. In the major portion of the investigation, after determination of dose level, pancreatic islets of 82 animals, 43 control and 39 alloxanized, were examined histologically. Of the 39 experimental animals, 3 failed to respond to alloxan. Islets from the remaining 36 animals were evaluated.

Blood Glucose Method

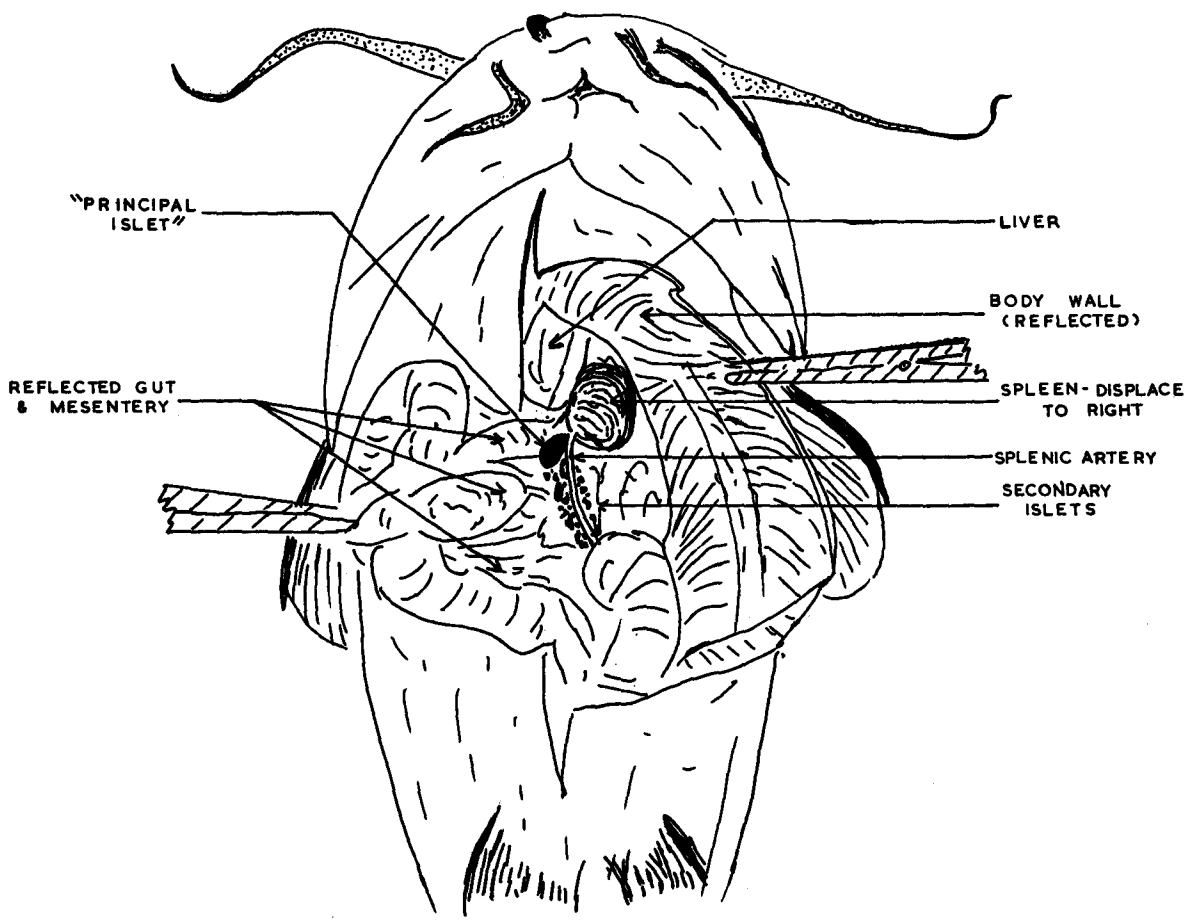
A modification of the Folin-Malmros micro method for the estimation of blood glucose was employed in the determinations reported in the present work. The procedure followed gives more adequate precipitation of fish blood proteins than do other methods tested. In addition, it is fairly rapid, and gives a linear relation between glucose concentration and logarithm of percent transmission of the final prussian blue colour complex in the range 0 to 200 mg % glucose.

The method is the result of some work done by the author in association with Dr. P.F. Nace at The Marine Biological Laboratory in the summer of 1957. Since the material has not yet been published, a summary is given in the appendix.

Histological Methods

Within 5 minutes of sacrifice, the principal islets of the fish were removed (Figure 2) and placed in Bouin's Fluid. Fixation was continued for 6 to 12 hours before the tissues were dehydrated for 2 hours in each of 3 changes of cellosolve (ethylene glycol monoethyl ether, Fisher, Histological Grade). They were then cleared in anhydrous benzene for 2 hours before infiltration in 2 changes, 20 minutes each, of molten 60°-63°C m.p. Tissuemat (Fisher) and embedded over an ice plate in similar Tissuemat.

FIGURE 2. SKETCH OF RELEVANT STRUCTURES IN THE ABDOMINAL CAVITY OF THE CATFISH.



A Lipshaw Tissue Processing Unit supplied agitation during fixation, clearing and infiltration.

Serial sections were cut at 5 microns and stained in groups of 18 either in Delafield's hematoxylin and triosin or in Scott's (60) variant of the Gomori aldehyde-fuchsin stain, with fast green FCF and phloxine as counter stains. Each group of 18 slides stained represented the tissues of about 15 fish, with slides of representative fish, both experimental and control, stained in consecutive racks in order to detect possible batch variations. All slides for staining were randomly selected from among experimental and control animals.

After staining, the sections were examined to determine changes introduced by alloxan administration. They were evaluated in terms of degree of beta granulation, nuclear morphology, and beta cell size. A study was also made of all the normal (control) animals, both from aldehyde-fuchsin and hematoxylin-triosin stained sections in an attempt to re-evaluate the normal histology of the catfish pancreas.

RESULTS

Determination of Dose Level

Twenty fish were injected with alloxan solution(asdescribed above) in doses ranging from 200 to 600 mg/kg body weight, after preliminary determinations of blood glucose level. The blood was then sampled and analysed for glucose 18 and 48 hours after injection. Table 1 (page 15) shows the results of these analyses.

The surviving treated animals, together with an equal number of control animals which had received intravascular distilled water, were sacrificed at 48 hours and their pancreatic islets examined histologically. These examinations, by the aldehyde-fuchsin technique, were in agreement with the expectations from the blood glucose response. Animals receiving 200 mg/kg showed no evidence of histological changes, while the 3 animals receiving 300 mg/kg showed varying degrees of degranulation. Those receiving 400 or 450 mg/kg showed more extensive partial degranulation, a few examples of hydropic degeneration and some pyknotic nuclei. The surviving animals receiving 500 mg/kg or more showed nearly complete, or, in one case, complete degranulation and extensive nuclear changes in the beta cells.

TABLE 1

Effect of Various Doses of Intravascular Alloxan on Blood Glucose in the Catfish

Alloxan mg/kg	# Fish	Experimentals (Alloxan) Blood Glucose, mg%			# Fish	Controls (Water) Blood Glucose, mg%		
		at inj.	18 hrs.	48 hrs.		at inj.	18 hrs.	48 hrs.
200	4	38	78	60	4	42	41	58
300	3	60	100	115	3	39	60	64
400	3	32	130	165	3	70	62	73
450	3	44	136	180[i]	3	40	49	38
500	3	55	138	156[i]	3	68	71	59
600	4	45	200[ii]	all dead	4	64	61	56

[i] These readings for 2 animals only; 1 died between 18 and 48 hours.

[ii] This reading for 2 animals only; 2 died before 18 hours.

The 400mg/kg dose was selected as satisfactory to produce diabetes in future experiments. It is characterized by a high survival rate, a persistent hyperglycemia and beta cell damage.

Subsequently 50 fish were treated with 400 mg/kg alloxan. Four of these failed to develop hyperglycemia. This indicates that the dose is diabetogenic at the 92% level.

Excluding the group used to determine response over periods up to 14 days, 45 animals were treated with alloxan and 45 with distilled water. Six of the alloxanized animals died before sacrifice (survival rate - 87%), as did 2 control fish (survival rate - 96%).

Blood Glucose Response to Alloxan

Marked hyperglycemia was characteristic of alloxanized fish examined 3 hours to 14 days after treatment. Glucose in blood samples taken 3 hours after treatment ranged from 88 mg % to 165 mg %, with a mean value of 121 mg %, more than twice the normal value. Of the 21 animals studied at this period, one showed no response to treatment. Hyperglycemia persisted with minor fluctuations through the remainder of the 14 day period studied.

Table 2 (page 17) summarizes the results of all the glucose determinations on 100 fish, 50 alloxanized and 50 control. Determinations on alloxan injected fish may be compared with the series

TABLE 2. EFFECT OF ALLOXAN ON BLOOD GLUCOSE IN THE CATFISH

TIME AFTER TREATMENT	ALLOXAN INJECTION			DISTILLED H_2O INJ.		
	No. OBS'NS	BLOOD GLUCOSE, MG %	S *	No. OBS'NS	BLOOD GLUCOSE, MG % †	S *
3 HOURS	21	121	28	21	63	5
6 HOURS	10	168	30	10	67	31
9 HOURS	11	124	29	11	58	15
12 HOURS	8	151	25	8	57	16
18 HOURS	15	167	34	12	65	23
24 HOURS	12	169	36	10	62	37
48 HOURS	13	140	12	14	59	21
72 HOURS	9	157	20	7	53	14
96 HOURS	8	150	15	7	73	20
5 DAYS	4	162		4	59	
6 DAYS	4	183		4	77	
8 DAYS	4	152	21‡	4	58	23‡
10 DAYS	3	188		2	81	
12 DAYS	3	178		2	74	
14 DAYS	2	178		2	80	

$$* \; S = \left\{ \frac{\sum_{i=1}^{N} X_i^2 - \frac{\left(\sum_{i=1}^{N} X_i\right)^2}{N}}{N-1} \right\}^{1/2}$$

† 237 ANALYSES OF UNTREATED OR H_2O INJ. FISH GIVES MEAN BLOOD GLUCOSE = 59 MG. % & S = 19

‡ FEW ANALYSES AT EACH STAGE; ONE "S" CALCULATED FOR ENTIRE GROUP.

carried out on control fish at the same time. The general response to alloxan treatment is more clearly shown in Figure 3 (page 19).

The standard deviation, "s", was calculated from the formula (9) given in Table 2, for each series of determinations and also for the complete group of "normal" blood sugars. The 237 determinations done on untreated or distilled water injected fish gave a mean value of 59 mg % and a standard deviation of 19 mg %. It will be noted that some of the standard deviations are large, particularly among the experimental groups. This is because all results, including 7 analyses from the 4 fish which showed no response to the alloxan treatment, were included in the calculations. The high values for "s" in certain of the individual control groups are the result of a few determinations at or beyond the usual maximum and minimum. These features are indicated in Figure 4 (page 20).

The apparent departure from a normal distribution shown (Figure 4) by the treated animals is explained by the fact that the method of blood glucose analysis used (see appendix) is applicable only to 200 mg %. Values above this level are interpreted as 200 mg%.

As a check on reproducability of the blood glucose analysis, duplicate analyses were carried out periodically on samples randomly selected from normal and treated fish. The 50 such

FIGURE 3· EFFECT OF ALLOXAN ON BLOOD GLUCOSE IN THE CATFISH

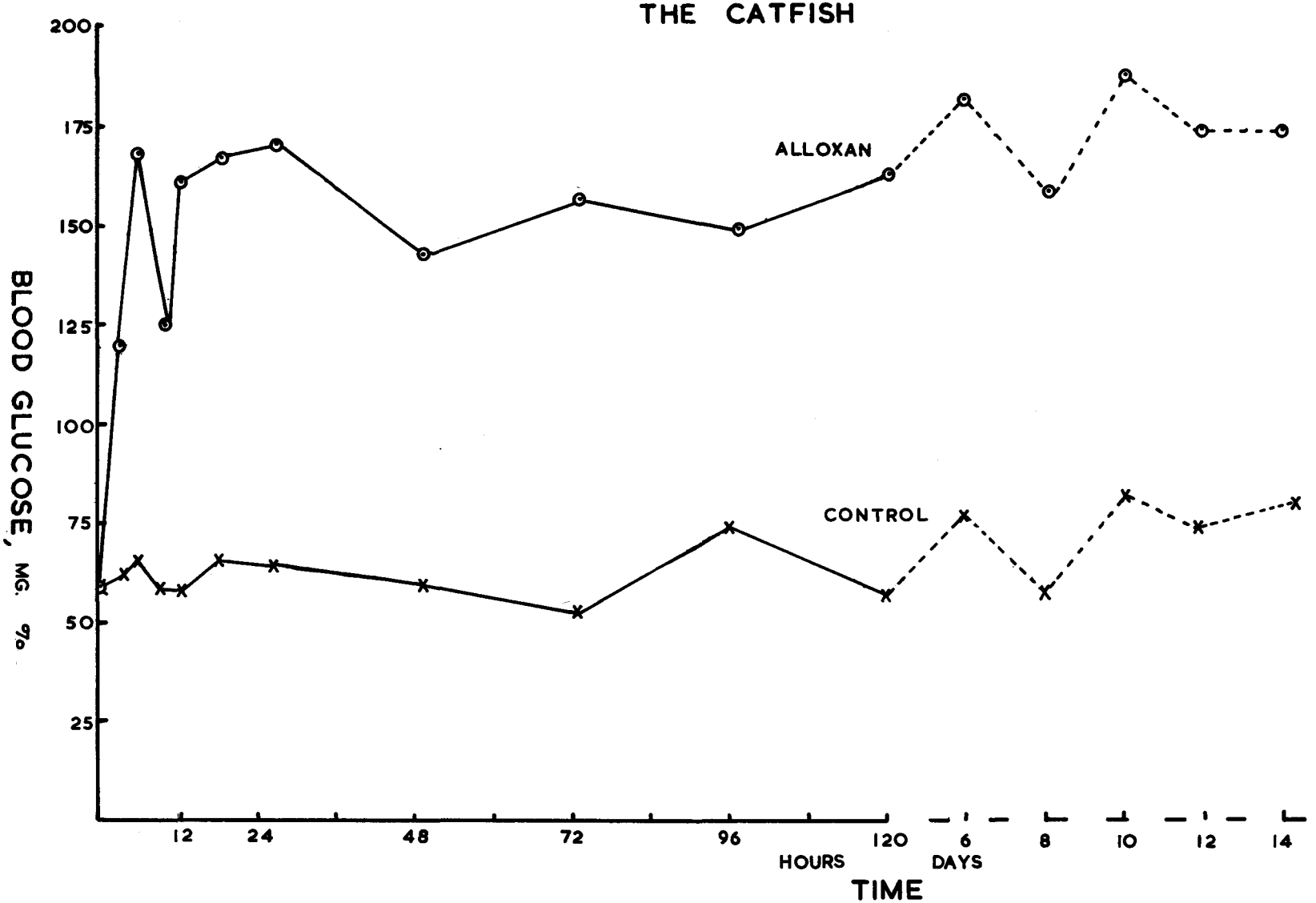
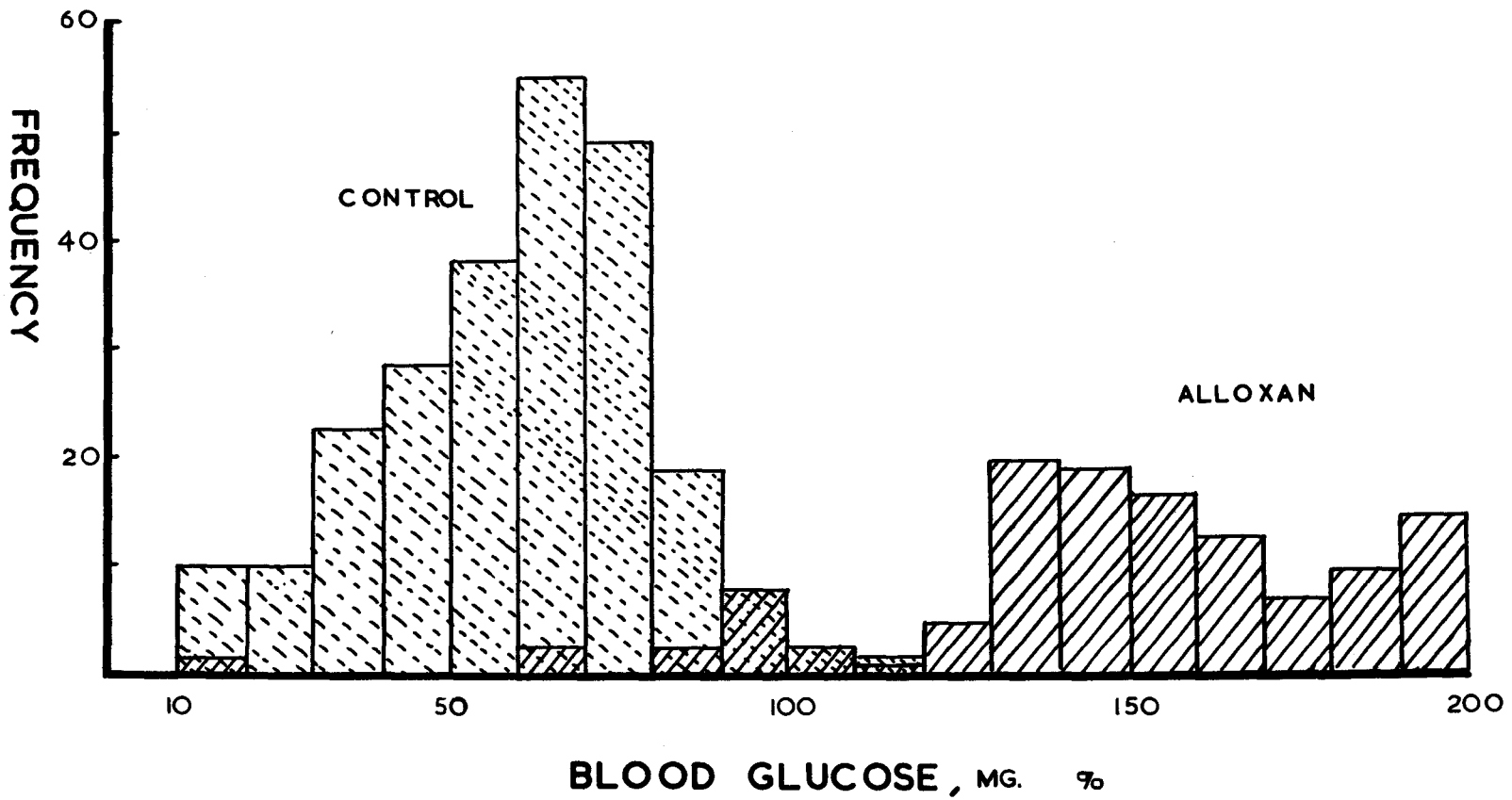


FIGURE 4. COMPARISON OF BLOOD GLUCOSE IN NORMAL & ALLOXAN INJECTED CATFISH.



analyses performed agreed to within 10 mg %.

Normal Histology of the Pancreas

This description is based on the examination of pancreatic islets from 43 normal fish, cut in sections at 5 microns. While none of the authors who have previously reported on this matter has specified the number of animals examined, the contradictions and certain other features of their treatments suggest that the numbers have been small. The present work is not regarded as a complete description of the catfish pancreas but rather as an analysis of the endocrine beta cell distribution in the population sampled.

In the catfish, as in many other teleosts, the endocrine pancreas occurs in clumps up to 3 millimeters in diameter in the mesenteric folds along the splenic artery. As suggested by McCormick (44), one of these (Rennie's (54) "Principal Islet"), is larger than the others and lies between the layers of mesentery near the proximal end of the splenic artery. In a few cases, 2 or even 3 such large islets were found. Twenty to 50 other islets, ranging in size from 1 millimeter diameter to groups of a few cells, visible only microscopically, are distributed along the length of the splenic artery. Bimes (4) suggests that islet tissue

is also present in the liver, while Vincent and Thompson (67) state that they have never detected islet tissue in the intrahepatic pancreas. We have not attempted to clarify this matter.

The sections reveal that the islets consist of both endocrine and exocrine tissue (Plate 1, page 23), with the endocrine in the largest proportion. The islets are usually encapsulated by a thin layer of connective tissue, which, however, is not universally present, especially in the smaller islets. Alpha and beta cells have been identified in the endocrine portion and there is evidence that some other unidentified cell types are present. The endocrine tissue is interrupted by deep invaginations of acinar tissue (Plate 1) which may, in single sections, appear as isolated areas surrounded by endocrine cells. This acinar tissue is frequently associated with connective tissue elements. The vascular supply, abundant in all regions, seems more plentiful in the endocrine portion (Plate II). No ducts have been identified in the acinar tissue occurring within the islets.

On staining with Gomori's aldehyde-fuchsin, it is apparent that beta cells comprise the largest portion of the endocrine tissue. These cells (Plate III) contain large numbers of aldehyde-fuchsin positive cytoplasmic granules. A phloxine counterstain

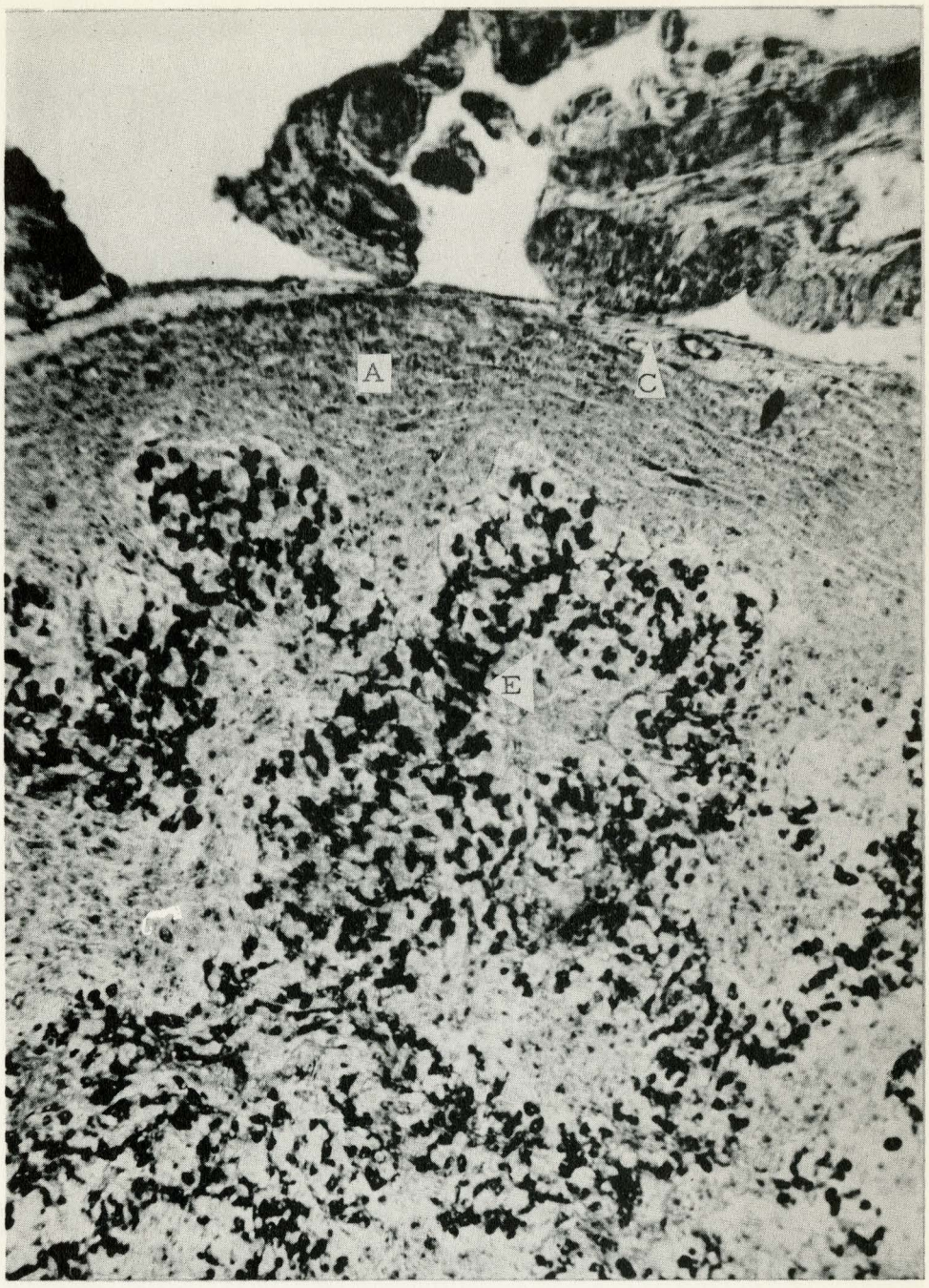


PLATE I

Normal Catfish Pancreas
Shows distribution of exocrine (A) and endocrine (E) elements and connective tissue capsule (C).
Aldehyde-fuchsin, X150.

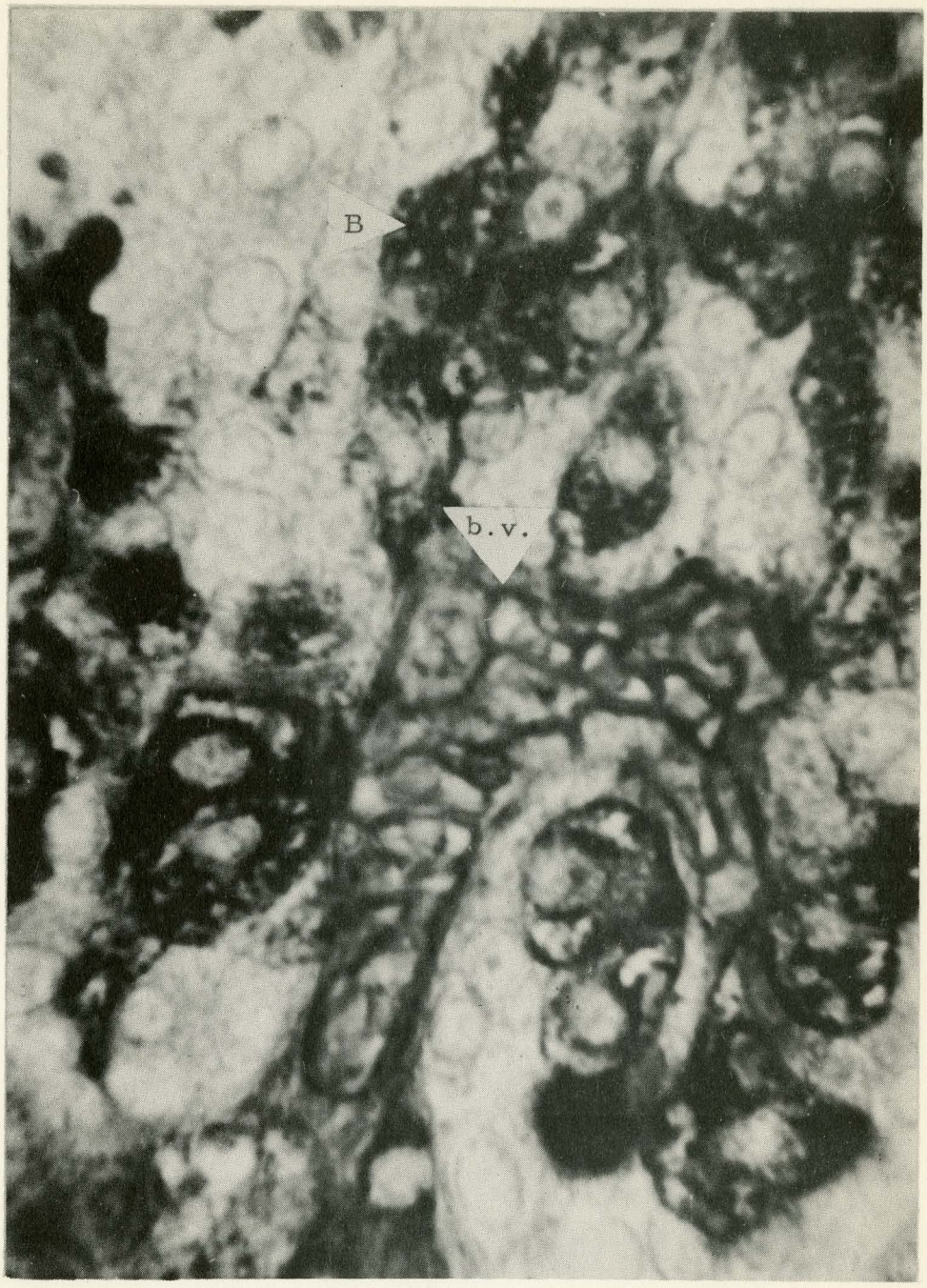


PLATE II

Normal Endocrine Catfish Pancreas
Engorged blood vessel (b.v.) and normal beta granulation (B). Aldehyde-fuchsin, X1500

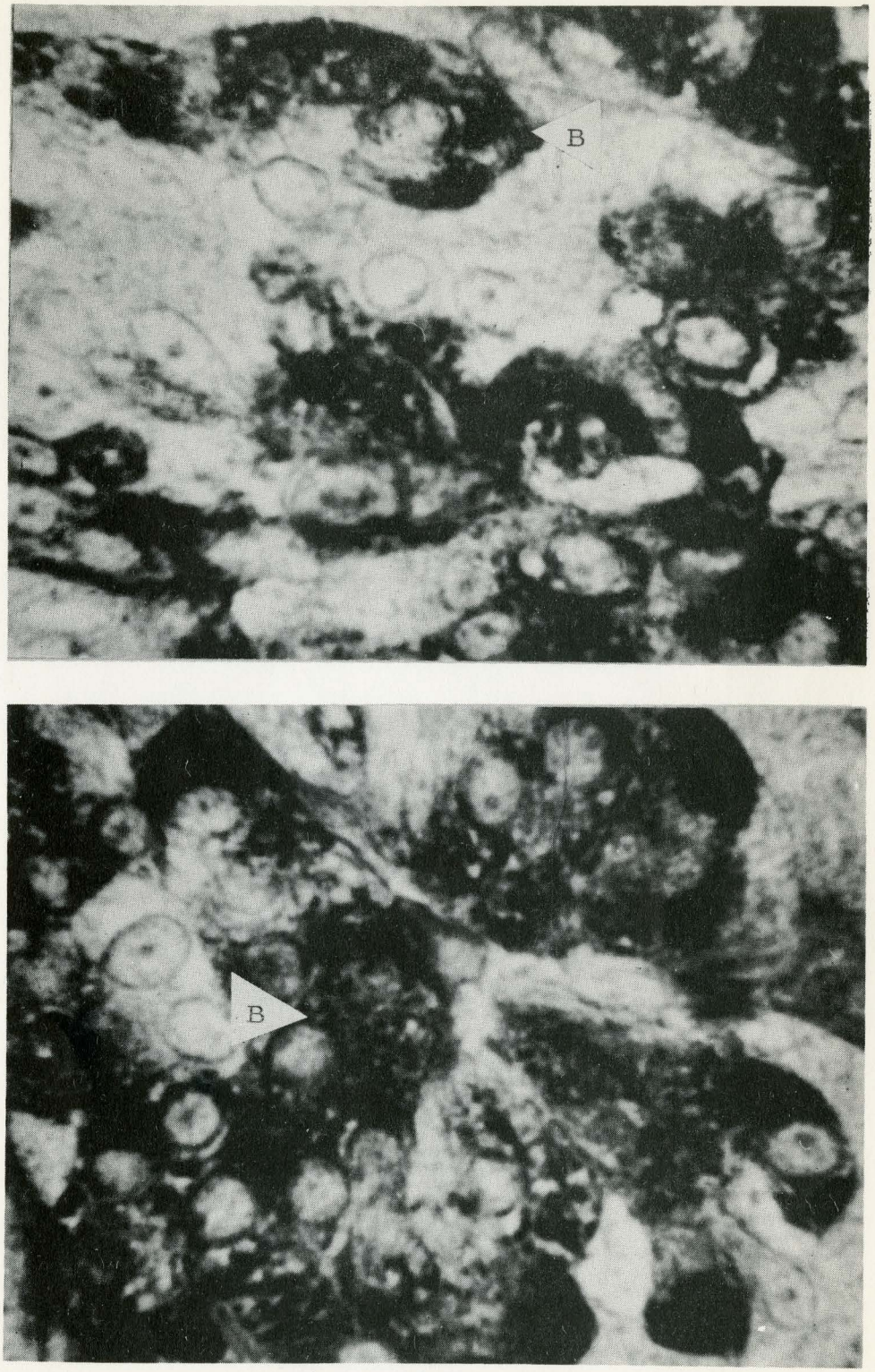


PLATE III

Normal Endocrine Catfish Pancrea
Shows range of beta cell distribution and norma
beta granulation (B). Aldehyde-fuchsin, X1500

shows a spherical nucleus containing a well defined nucleolus. On a few sections, alpha cells were demonstrated by the modification of Cason's trichrome stain suggested by Rona and Morvay (56). Consecutive sections stained by Gomori's method and that of Rona and Morvay indicate that some of the cells in the endocrine portion cannot be classified either as alpha or beta.

The islet capsules are typically surrounded by acinar tissue, (Plate 1), the amount being approximately inversely proportional to the size of the islet, ranging from a few cells to a layer a millimeter or more thick. Thus the smaller, typically unencapsulated islets are apparently embedded in a mass of exocrine tissue, as in the mammal. Prominent pancreatic ducts may be seen in these heavy layers of acinar tissue.

Effects of Alloxan on Pancreatic Histology

Sections from treated animals were readily distinguished from normal tissues. The typical response to alloxanization is partial degranulation, evident as early as 3 hours after treatment. The tendency progresses towards total degranulation accompanied by nuclear breakdown and cytoplasmic degeneration at later stages. No similar changes occurred in the control animals.

The 4 fish whose blood glucose showed no change after alloxan did not undergo any histological changes. They are not included in the following evaluation.

In general, histological response paralleled the blood glucose changes. Some beta degranulation was apparent as early as 3 hours after alloxan, and in an intensified state this degranulation continued throughout the entire period studied. Total degranulation occurred in 2 of the 4 animals studied at the 96 hour period. In classifying these changes, an arbitrary numerical scale of granulation from I (normal) to IV (totally degranulated) was established (Plate IV).

Some examples of hydropic degeneration, with sparse granulation restricted almost entirely to the periphery of the cells were seen. Pyknotic nuclei were common in stages from 48 hours, and some were seen earlier. A few small nuclei, noted close together in cells showing no granulation may be late telophase stages of mitotic division, but the number of animals in which this was observed is not adequate for a complete evaluation of this possibility.

The general features of the histological changes (19) are outlined in Table 3, and illustrated in Plate IV.

TABLE 3

Histological Effects of Alloxan on the Pancreas

Stage Sacrificed	Beta Granulation*	Other Features
3 hours	4 - II 1 - III	None
6 hours	1 - II 3 - III	Hydropic degeneration in 1; pyknotic nuclei in 2
9 hours	5 - III	pyknotic nuclei and peripheral granulation in 1
12 hours	1 - II 4 - III	slight cytoplasmic disintegration; pyknosis in 2
24 hours	1 - II 4 - III	cytoplasmic degeneration in 4
48 hours	1 - II 1 - III 2 - IV	pyknotic nuclei in 4 "telophase" figure in 1 hydropic degeneration in 2
72 hours	2 - II 2 - III	pyknosis and "telophase" figures in three; extensive cytoplasmic degeneration
96 hours	2 - III 2 - IV	general breakdown in 2 at IV; others, pyknotic, telophase figures in 1

*Arabic numeral indicates number of fish observed; Roman numeral degree of granulation, from I(normal)to IV(total degranulation).

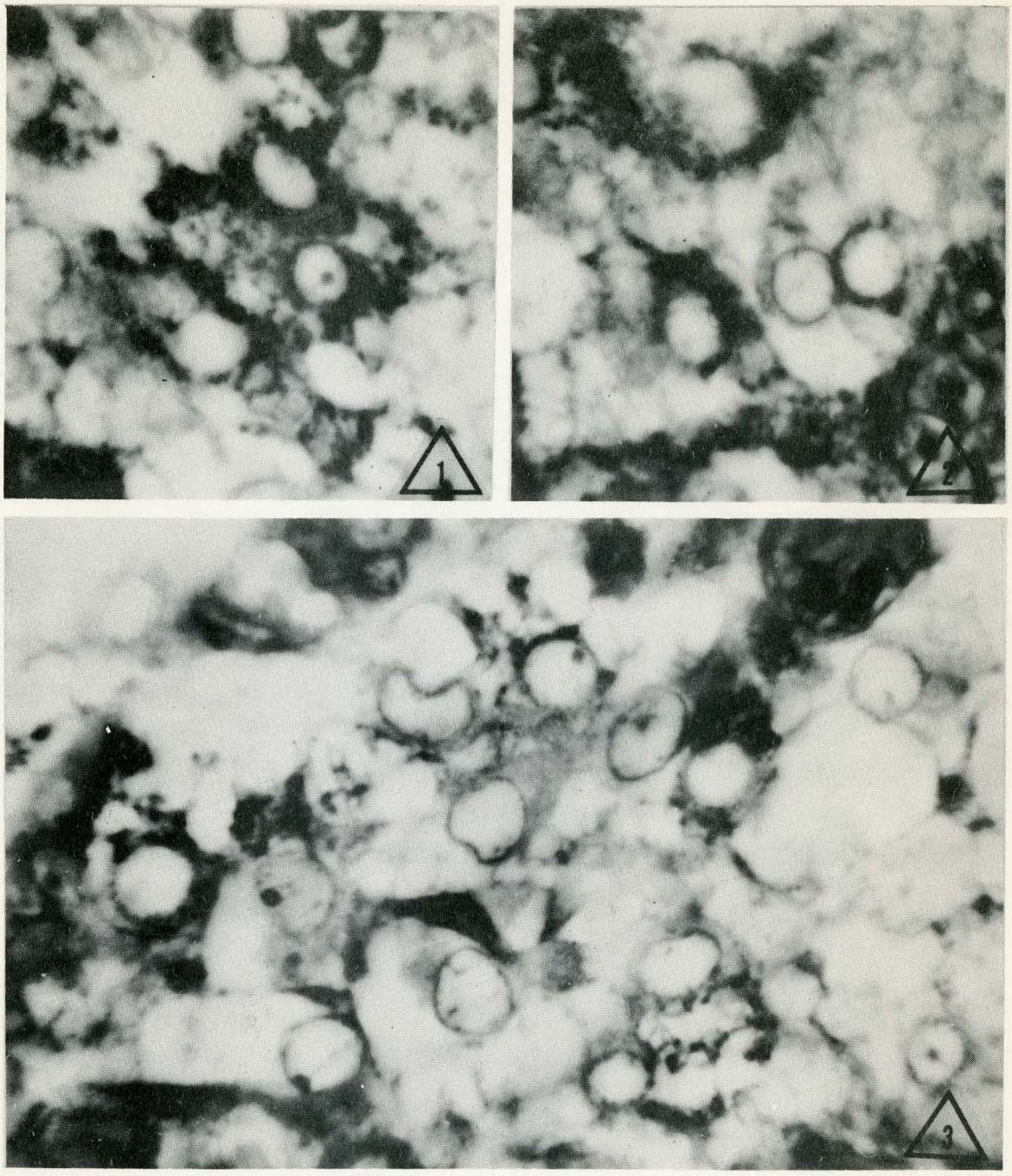


PLATE IV

Pancreatic Changes After Alloxan

1. Stage II degranulation and cytoplasmic degeneration.
2. Stage III degranulation.
3. Stage IV degranulation, with cytoplasmic disintegration and nuclear degeneration

All Aldehyde-fuchsin, X1500.

DISCUSSION

This investigation has given detailed data concerning normal and alloxan diabetic blood chemistry and pancreatic histology in the catfish. The study was undertaken from the dual viewpoints of biochemistry and histology in order to elaborate existing knowledge of certain features of tissue interaction and maintenance as exemplified by the endocrine pancreas. In addition, it extends a more precise understanding of blood glucose regulation to another group of teleosts. As an analysis of the response of another vertebrate to a particular diabetogenic treatment, the investigation may be regarded as a contribution to comparative physiology. It is possible that experiments based on the suggestions advanced below will provide information of even greater significance than that given by the present study.

Along with the specific information, the work has given indication of the usefulness of this experimental animal for investigations of this nature. The conus arteriosus, suggested by Simpson (63) as a site for blood sampling, has proven ideal for direct intra-arterial injection. Administration of alloxan by this

route insures it rapid distribution throughout the organism, including the prominently vascular endocrine pancreas. This appears important because of the report of Patterson, Lazarow and Levy (53) in which the effective half life of alloxan has been estimated at 1 minute, under conditions of temperature and pH similar to those found in animals. This site has shown no serious damage from injection and repeated sampling, and has proven much more useful than the gill arch used in the toadfish (48).

The general hardiness of the animal has been of importance in several aspects of the work. The ability of the catfish to withstand anoxia has already been demonstrated by Simpson. The survival of the fish without food through several weeks has been helpful in maintenance of a comparable basal state among the members of the experimental group and in keeping stocks of animals through the winter. The fact that the animal responds less vigorously than many fish to removal from water has been convenient in allowing handling without anaesthesia or special holding devices. Improvements in water supply, temperature control, feeding, and skin prophylaxis should permit use of the animals for studies of longer duration.

Another outcome of the work more general in its application than the specific data obtained has been the blood sugar method used. The experience of this investigation has demonstrated that

the method is characterized by a high degree of reproduceability, sensitivity to ranges as low as 15 mg% and avoidance of some difficulties found in some other methods.

The normal blood sugar values found were markedly more variable than those seen in mammals and birds, but much more uniform than the values reported for the toadfish (39, 48, 49). The mean values for the two species were very similar, and compared favorably with the values reported by Simpson for catfish and flounder. The value given in The Handbook of Biological Data (65) for the only fish cited, the carp, is nearly twice the value found in the catfish, toadfish and flounder. The significance of this difference cannot be evaluated without knowledge of the method used for the carp.

The changes in blood sugar level seen after alloxanization were large enough to be considered definite departures from the normal range, the mean value after treatment being approximately twice the normal value. This magnitude of hyperglycemic response, in percentage of normal value, is equal to or exceeds that reported for the common laboratory mammals. A confused picture is presented by the data available for the only other fish subjected to comparable study. Using venous or capillary blood from the tail of the toadfish, Lazarow (39) found a substantial hyperglycemic response after subcutaneous alloxan. Study of arterial blood taken

from the gill arch by Nace (48, 49) indicated a less marked or very variable response.

The significance of the histological study of normal tissues derives from the fact that previous reports antedated modern methods for staining of beta cells. The presence of both exocrine and endocrine components in the islets has been confirmed, and the endocrine element has been analyzed more adequately. While the endocrine element is predominant, enough exocrine tissue is found to preclude the possible use of this material in metabolic studies of isolated endocrine tissue. The fact that histological changes paralleled blood glucose values supports the accepted view of beta cell function in insulin synthesis.

Changes similar to those seen in alloxanized catfish have been reported by Lazarow (39) for the toadfish and by Grosso (23) for the guppy. Differences between the methods and findings of Lazarow and of Nace (48) have already been discussed. The situation in the guppy is complicated by the known chemical instability of alloxan. This suggests that some mechanism more complex than direct action of the dissolved alloxan may have been involved in the work of Grosso.

Even the mechanism of the direct action of injected alloxan is not well established. It is now generally accepted that alloxan diabetes is pancreatic in origin, resulting from the direct effect of

alloxan on the beta cells, though the actual mechanism of this action is still open to question. The most widely held theory is Lazarow's glutathione hypothesis (53) which suggests that alloxan combines with essential sulfhydryl groups of enzyme molecules, effectively poisoning them and resulting in cell death. A lower glutathione concentration in beta cells than that present elsewhere explains the selectivity of alloxan action on beta cells. The fact that higher doses of alloxan are toxic to other cell types, and even to whole animals, supports the hypothesis, as does the fact that injections of glutathione, if properly timed, can protect animals from alloxan diabetes (37). Consideration of the low metabolic rate of the fish suggests that study of the pancreas and other organs, especially the liver and kidney, immediately after the injection of alloxan may provide evidence relevant to the Lazarow hypothesis.

This preliminary analysis of alloxan diabetes in the catfish leaves several questions to be answered. Histological analyses should be conducted beyond 96 hours, as well as extended to a larger number of animals at the earlier stages. The significance of the possible "telophase" figures could be more completely understood after studies with colchicine and tritium labelled thymidine. An analysis of the early cell changes by similar methods might give evidence of the mechanism of initiation of islet

damage, which is completely unknown at present. The metabolic status of partially or totally degranulated beta cells in insulin production might be elucidated by autoradiographic studies after treatment with isotope labelled insulin precursors, such as S^{35}cysteine.

Such studies would undoubtedly cast more light on the function of alpha cells. At present one of the greatest difficulties in studying alpha cells is the lack of completely adequate methods of staining them. The answer to this problem might be found in autoradiography with isotope labelled glucagon precursors.

The relationships between Opsanus and the catfish in terms of the present study need more elaboration. The adequacy for both forms of the blood glucose method indicates similarities in the general features of blood chemistry. However, the lack of uniform response to alloxan noticed in the toadfish is indicative of fundamental differences. The response to steroids, which has suggested diabetic-like conditions in Opsanus (49) should be investigated in the catfish. Also of interest would be studies of the effect of tolbutamide on diabetic catfish.

At the present, there appears to be no single critical experimental approach capable of clarifying the general relationship between other pathological conditions, such as cancer and cardiovascular disease and diabetes, or capable of casting light on the

general features of tissue growth and interaction as exemplified by pancreatic regeneration. Far more extensive examination of these relationships is needed. For such work, the catfish and other poikilotherms present many important advantages. The exploitation of these advantages may be a significant technical advance toward improved understanding of tissue interaction.

SUMMARY

1. The common lake catfish, Ictalurus nebulosus, has anatomical features, particularly the location and structure of conus arteriosus and pancreatic islets, suitable for investigation of experimental diabetes.
2. The islet tissue includes both exocrine and endocrine elements, with beta cells a major constituent of the endocrine tissue.
3. Blood glucose, determined by a new modification of the Folin-Malmros micro procedure, had a mean value of 59 mg% and a standard deviation of 19 mg%. These data are based on 237 blood glucose determinations on 100 normal catfish.
4. Alloxan-treated catfish showed hyperglycemia within 3 hours, with levels of 121 to 188 mg% maintained to 14 days.
5. Pancreatic beta cells of treated fish were partially degranulated within 3 hours. More severely damaged cells were found at later periods, with complete degranulation present in some cases after 48 hours.

APPENDIX

Determination of Blood Glucose

In the past fifty years many methods have been designed for the estimation of blood glucose. Typical of the earliest methods is that of Eddie and Spence (14), who based an analysis on the reduction of a known quantity of Fehling's solution by 50 millitres of dialysed blood, followed by a gravimetric determination of the resultant copper oxide. The same principle is used in some of the more recent methods, but refinement of chemical techniques now permits determinations in minutes rather than days, and the use of as little as 0.01 millilitres of blood.

Several principles have been used in the determination of glucose in biological fluids (12, 16, 57, 66), but the most commonly used ones are based on the ability of hot alkaline solutions of glucose to reduce certain metallic ions, usually cupric or ferricyanide ions (28). The extent of the reduction is then measured by colorimetric (16, 50), gasometric (66), volumetric (12), or gravimetric (54) means.

Since some non-glucose reducing substances, probably

largely glutathione and glucuronic acid (15), are present in blood, various methods of analysis give different values, depending on their degree of specificity for glucose. Yeast fermentations are the most specific (28), but technical difficulties preclude the use of the method on any large scale. The high cost of reagents has limited the recently proposed enzymatic methods. As the reduction methods are carried out on "deproteinized" blood, the efficiency of the deproteinization procedure in removing non-glucose reducing substances is at least partially responsible for the overall specificity of the determination.

Several hundred glucose analyses on the blood of two species of fish (toadfish, (Opsanus tau) and catfish, (Ictalurus nebulosus)) have convinced the author that the methods of deproteinization recommended for clinical use (24, 28), are unsatisfactory when applied to the blood of fish. This is not surprising, since these methods have been designed for clinical use and can hardly be expected to be adequate unless restricted to human blood, or at least mammalian blood.

Simpson (63) has suggested the possibility of the existence of "some protein - sugar 'compound' or polysaccharide in the blood" of the catfish, since he found higher values for blood glucose after hydrolysis of blood samples. The hypothesis found

further support by the finding that after asphyxia, hydrolysed and unhydrolysed blood samples were approximately the same in glucose level. This possibility of a "sugar containing" (i.e., reducing) substance in catfish blood makes it essential that any deproteinization procedure used must be very efficient if "true" glucose values are to be obtained.

It is recognized that tungstic acid precipitation of blood proteins, clinically at least, (28), gives values higher by about 20 mg% than yeast fermentation studies. There is adequate evidence that this deviation does not change appreciably either during hyperglycemia or hypoglycemia. The determinations on which these statements are based employ blood and tungstic acid in the ratio 1 : 10.

In the method presented here for the determination of glucose in fish blood, precipitation was carried out with the blood acid ratio at 1 : 100. The increase in amount of acid gives a more rapid and complete precipitation of proteins.

In addition, we have used Duponal, as recommended by Horvath and Knehr (29) rather than gum ghatti (16) to keep the final prussian blue complex in suspension. The gum ghatti is undesirable because it makes it necessary to add potassium permanganate daily to remove foreign substances.

The concentration of potassium ferricyanide has been

adjusted to be compatible with the absolute amount of glucose present in the ranges of bloods being measured. This also reduces the interference of yellow ferricyanide colour with the prussian blue suspension whose colorimetric determination makes the analysis quantitative.

The final refinement consists of a daily calibration curve, prepared by using a series of standard glucose solutions in place of blood in one series of tubes. These standards, carried through the entire procedure at the same time as the unknown bloods, completely eliminate the possibility of batch variations.

Details of Analysis

I Preparation of Reagents

(1) Standard Glucose Solutions: Solutions of 0, 25, 50, 80, 100, 150, and 200 mg% in saturated benzoic acid are prepared volumetrically from a solution of 5.0000 grams glucose (Analar) in 500.00 mls solution.

(2) Dilute Tungstic Acid: With continuous agitation, add simultaneously, to 480 mls distilled water, 10.0 mls sodium tungstate and 10.0 mls 0.67 N sulfuric acid.

N.B.: Sodium tungstate solution must be balanced to pH 7.0 by addition of 1 N hydrochloric acid.

(3) Potassium Ferricyanide Solution: Dissolve 250 mg. potassium ferricyanide (ferrocyanide free) in 500 mls distilled water. Store in brown bottle; discard if

solution turns blue on addition of Ferric Duponal reagent or on formation of a precipitate.

(4) Buffer Solution: Dissolve 4.0 grams anhydrous sodium carbonate in 20-25 mls distilled water; add 75 mls freshly prepared 1.0% sodium cyanide; dilute to 500 mls. This solution must be prepared fresh daily, and has a pH between 10.5 and 11.0.

(5) Ferric Duponal Solution: Dissolve 0.10 g crystalline ferric ammonium sulfate in 10-15 mls water; add 5.0 mls 85% phosphoric acid. Add 0.30 g Duponal dissolved in 30-40 mls water; dilute to 100 mls.

II Procedure

(1) Bubble 0.1 mls blood or glucose standard into 10.0 mls tungstic acid in centrifuge tube.

(2) After allowing to stand at least 15 minutes, centrifuge 5 minutes.

(3) Pipette 1/2 ml supernatant into photometer tube.

(4) Add 1/2 ml potassium ferricyanide reagent.

(5) Place in boiling water bath, 15 seconds.

(6) Add 1/2 ml buffer solution.

(7) Cover tubes with marbles, to prevent evaporation, and place in boiling water bath for 15 minutes.

(8) Cool to 25°-30° C. in water bath, (temperature meas-

ured in control tube containing 2 mls water).

(9) Add 1.0 ml Ferric Duponal Reagent.

(10) Add 3 mls distilled water.

(11) Wait ten minutes, then read in colorimeter at 630 - 640 $\mathring{A}$. Color is stable from ten to forty minutes.

Table 4 gives a typical series of experimental determinations, and the data for the calibration curve which appears as Figure 5.

Note that the calibration curve is an essentially straight line when plotted as the logarithm of percent transmission.

TABLE 4

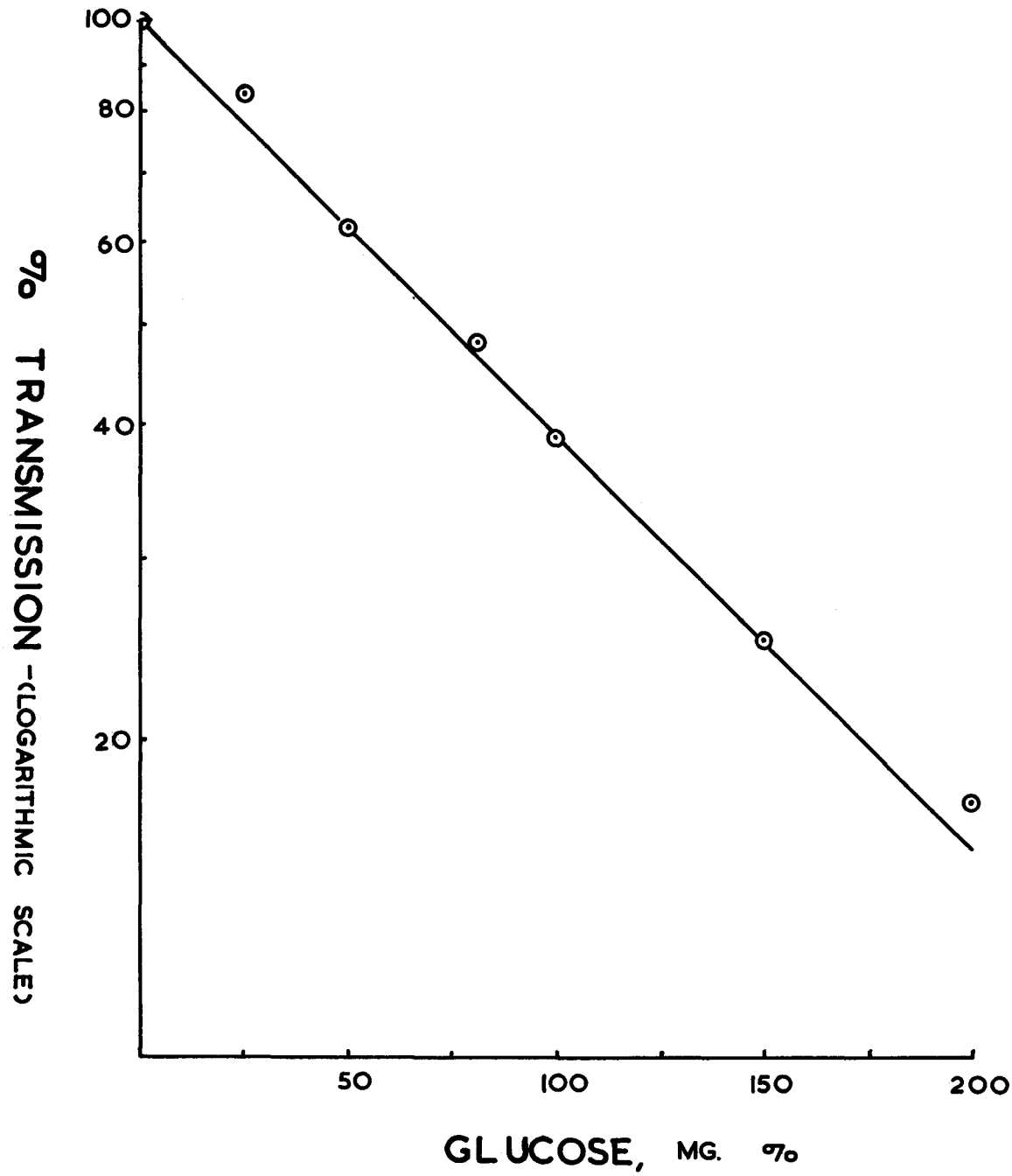
Typical series of blood sugar determinations on a group of fish 48 hours after injection.

Fish #	% Trans.	Glucose mg%	Treatment
94	27	142	Alloxan
95	24	155	Alloxan
96	50	74	Control
97	22	170	Alloxan
98	25	152	Alloxan
99	62	51	Control
100	38	104	Alloxan
101	71	38	Control
102	62	50	Control
103	58	57	Control

Calibration Data

Std. mg%	% Trans.
0	100
25	86
50	61
80	48
100	39
150	26
200	19

FIGURE 5. TYPICAL CALIBRATION CURVE



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