FISH BLOOD

REDUCING SUBSTANCES

REDUCING SUBSTANCES IN THE BLOOD

OF

NORMAL AND ALLOXAN TREATED FISH

By

MARGARET LAURA MOULE, B.Sc.

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TITLE: Reducing Substances in the Blood of Normal and Alloxan
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AUTHOR: Margaret Laura Moule, B.Sc. (McMaster University)

SUPERVISOR: Professor Paul F. Nace

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SCOPE AND CONTENTS: Blood sugar levels were determined in toadfish
and catfish by the Folin reducing procedure and a specific enzymatic
glucose procedure. The difference between the two values was con-
sidered to be the rest reduction (RR). After alloxan injection, the
RR level increased greatly reaching a peak at one hour and then
decreasing. The glucose level rose more slowly.

An attempt was made to study these changes in blood by paper
chromatography using silver nitrate to detect reducing compounds.
In normal blood, glucose, an unidentified compound, and several other
reducing compounds present in small amounts were separated. The large
amounts of RR present after alloxan could not be detected by silver
nitrate.

The significance of the results was discussed and future
investigations were suggested.

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Preface

A study of alloxan diabetes in fish has been conducted for a number of years by Dr. Paul F. Nace. During this study, unexpected changes in the blood rest reduction were observed after alloxan injection. This investigation represents the beginning of a study of these blood rest reduction changes.

The valuable experience gained from working with other students who have assisted Dr. Nace with different aspects of his alloxan diabetes research is acknowledged. The suggestions and support of Mr. John W. Moule and Mr. Paul E. Morrison are especially appreciated.

This investigation would have been impossible without the assistance and supervision of Dr. P.F. Nace. His kind help and encouragement are very gratefully acknowledged.

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Introduction

Alloxan has been used to produce hyperglycemic animals for diabetes research for nearly twenty years. Despite this, its action and the results of this action on carbohydrate metabolism are not understood. It is clear, however, that alloxan does more than just produce hyperglycemia. Recent studies reveal that the results of its action are more complex than originally appeared and indicate a great deal is yet to be learned. It is essential that we understand its action and results while its use is so widespread and so important. Such knowledge may also assist our understanding of diabetes.

The original view of the results of alloxan action is quite simple. Alloxan (pyrimidinetetrone) when injected into animals appears to damage the beta cells of the islets of Langerhans in some manner. The islets consist mainly of beta cells which produce insulin. With their destruction (after the right dose of alloxan), hyperglycemia results, presumably because insulin is no longer produced. This is usually studied by a histological examination of the islets accompanied by blood sugar determinations. Such studies of alloxan diabetes have been made using mammals, birds, reptiles, amphibians, and fish. Review articles have appeared by Bailey (6), Lazarow (45), and Lukens (50). Fish have proven especially valuable because of their lower rate of metabolism and their separate islet tissue. Alloxan diabetes in fish (essentially similar to that in mammals) has been studied by Lazarow and Berman (46), Nace (59), Murrell (55),

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and Falkmer (25). These investigations while showing blood sugar and pancreatic changes have indicated that other changes occur after alloxan.

That other changes do occur is well illustrated by our results. During a study of alloxan diabetes in Opsanus tau, it was unexpectedly found that a large amount of non-glucose reducing material appeared in the blood. One hour after alloxan, in some cases there was ten times as much non-glucose material as glucose. Normally there is less than twice as much (61). These results were very striking.

Although it has been known for many years that such non-glucose reducing material, also called the rest reduction, RR, (29) or saccharoid fraction (9) is present normally in blood, little is known of its exact nature or function. It is measured by comparing the value of the reducing power of blood (or urine) with a more accurate value for glucose only. The former may be determined by a reducing procedure such as that of Folin and Malmros (28) and the latter by fermentation (2) or enzymatic (69) methods or less quantitatively by chromatography (49). The unexpected appearance of this large quantity of non-glucose reducing material, RR, after alloxan injection certainly warranted further examination.

This problem has several aspects and raises several questions. What is this material? Is it one or several substances? Is this material after alloxan injection an increase in the materials normally present in blood or is it something new? Where does it come from? When does it appear and how long is it present? What is the role of alloxan in its appearance? This problem is obviously very interesting

and the answers to these questions may be significant in an understanding of alloxan diabetes.

The presence of non-glucose reducing material has been shown by clinical studies. In human blood, the level of reducing substances is 80-120 mgm % (mgm/in 100 ml blood), but of this only 60-90 mgm % is actually glucose. About 25% then is RR (30). The amount varies from individual to individual, and within the same individual under different conditions (1). Certain compounds found in the blood are known to be reducing. These include fructose, amino acids, glutathione, phosphorylated hexoses, glyceraldehyde (53), uric acid, creatinine, glucuronic acid, dissacharides, purines, adrenalin (38), glucosamine and creatine (49). If urine is tested the list may be extended to include galactose, pentoses, glucuronides of some drugs, homogenistic acid (30), lactose, xylose, and arabinose (80). Strange, Dark, and Ness (76) found that the amino acids tyrosine, cystine, tryptophan, proline, hydroxyproline, and methionine interfere in reductometric methods. Certainly there are many substances which could be involved. The problem is to find out which ones are involved and in what way.

The behaviour and composition of the rest reduction in normal blood have been studied. Folin and Svedburg (29) in 1926 studied the RR in humans. They found it dropped after insulin injection even more sharply than glucose. After adrenalin its increase was relatively slower than that of glucose, but it did increase. They concluded that the RR represented an unknown phase of carbohydrate metabolism.

Benedict (9) in 1931 used the term saccharoids for the RR of carbohydrate nature and presented evidence as to why he felt little of it was glutathione. Fashena later stated that one-third of it was glutathione (26) and the other two-thirds glucuronic acid or its compounds. Only a very small part was due to uric acid, creatinine, and ascorbic acid (27). Apparently then the major part of the RR in normal blood is considered to be glutathione and glucuronic acid and its compounds.

The behaviour of the RR in diabetic patients has been studied in a few cases. Hiller, Linder, and Van Slyke (38) in 1925 found that the higher the blood sugar, the greater was the difference between the reducing and true glucose results. Jakobsen (41), on the other hand, found no correlation between blood glucose and RR levels, although variations in the RR level occurred during glucose tolerance tests. Baumgarten (8) using paper chromatography concluded that the amount of other sugars present frequently agreed with the severity of the disease. He found fructose and lactose in diabetic blood as well as glucose and other unidentified sugars. Lohman (49) also using paper chromatography found that in 53% of his diabetic patients, the RR increased along with the blood glucose, but only in 17% did the percentage of RR increase over that of glucose. He did not identify the material found besides glucose. These results while not conclusive indicate that the RR may be significant in diabetes.

Considerable work has been done on the RR of fish. White (79) in 1928 using fermentation studies found that while the RR is insignificant in elasmobranchs, it is present in appreciable amounts in

teleosts, 10-12 mgm %, a large fraction for fish. McCormick and
MacLeod (52) in 1925 suggested that masked carbohydrates might be present
in fish blood when they found that heating the blood in the presence of
HCl increased its reducing power. This increase was greater if whole
blood rather than a protein-free filtrate was used. They suggested
that the hyperglycemia which they found after handling might be due to
these masked carbohydrates. Simpson (73) in 1926 on hydrolysis of
whole blood increased its reducing power two to three times. He
suggested that these masked carbohydrates might be responsible for
asphyxia hyperglycemia. However, he decided against this theory when
he found in 1929 (74) that the liver must be present before asphyxia
hyperglycemia occurs. This early work indicated that the rest reduc-
tion might be quite important in fish.

More recent studies on the behaviour of the RR in fish have also
been made. Al-Gauhari (2) reported that Florkin found the RR un-
affected by asphyxia while Kiermeir found an increase in the RR after
exhaustion, during anoxia, and a slight increase after starvation.
Using fermentation methods, Al-Gauhari found that the RR disappeared
after feeding and did not reappear until 24 hours later. He found
asphyxia hyperglycemia involved a rise in true glucose. His values
for the RR were 32-38 mgm % which was 40-47% of the total reducing value
of 74-84 mgm %. Falkmer (25) found high values for the RR. The dif-
ference between the Hagedorn-Jensen method (supposed to be more accurate
than Folin) and glucose oxidase values amounted to 300 mgm % after
isletectomy or glucose injection. Great variations in values were

reported.   Normally the RR was about 25 mgm %, but after insulin injec-
tion it dropped to less than 10 mgm %.   Falkmer believes that the RR
is of a carbohydrate nature since non-carbohydrate reducing compounds
would not be likely to behave so under these conditions.   These results
indicate how little is known of the nature and role of the rest reduction.

There are many areas of carbohydrate metabolism in fish not
well known.   In a recent review of the subject, it has been pointed out
that most of the available information deals with glycogen, glucose,
lactate, and pyruvate (12).   Many of the studies have been concerned
with the factors which influence the blood glucose level.   This is
affected by anoxia, diet, water temperature, activity (44), sex and
spawning (43), as well as various experimental conditions.   These
complicate the study.   There are many things still to be learned.
The presence and significance of other carbohydrates are still to be
determined.   For instance, it has only recently been found that tre-
halose is an important component of carbohydrate metabolism in arthropods
(71) and other invertebrates (24).   In insects, it is a major transport
intermediate in glucose metabolism.   Similarly in fish with the applica-
tion of the latest techniques, the presence of other carbohydrates will
undoubtedly be detected.   The RR which has never been elucidated,
although its presence has long been known, demonstrates the need for
such studies.

If the literature of carbohydrate metabolism provides little
help in answering the questions about the RR, that on alloxan provides
none at all.   No mention of the role of alloxan in the production of
an increase in the RR was found.   Since this increase was most marked

at one hour and lasted less than 24, alloxan action during this period was studied. A number of reactions due to alloxan occur soon after its injection. An examination of these may help in understanding the increase in RR.

The rapidity of the alloxan action has been observed. Alloxan acts on the islets in the first five minutes after its injection. Gomori and Goldner (35) clamped the blood vessels to the tail of the pancreas for 5 minutes during and immediately after alloxan injection and then released them. They found the islets of this region un- affected while the rest were damaged. Archibald (3) determined that intravenous injected alloxan cannot be detected as alloxan in the blood after five minutes. He described three methods by which alloxan may be neutralized in the blood: by conversion to alloxanic acid by alkali, by conversion to alloxantin and then to dialuric acid by thiol groups, and by combination with urea. It has also been determined that the half-life of alloxan at pH 7.4 is 2.2 minutes (65). Also indicating alloxan's rapid action is the fact that substances which protect against alloxan damage, such as cysteine and glutathione, must be injected either before or within a few minutes of alloxan to be effective (11, 50, 66). Furthermore, histological islet changes can be observed after only five minutes (7). Alloxan itself then is not likely to be a part of the RR although some of its derivatives may be.

This rapid disappearance of alloxan is very likely connected to the rapid disappearance or decrease of glutathione at the same time. The blood glutathione is found mainly in the red blood cells. After

alloxan, the glutathione level drops and does not rise to normal for
several hours (11, 47, 66). This has been found in fish as well.
Falkmer (25) determined that it drops increasingly with increasing
alloxan doses. This suggests that glutathione is probably not involved
in the alloxan RR.

A very striking effect is produced by alloxan on the red blood
cells. Berardi and Gordon (10) found a drop in the red blood cell count
and hemoglobin count occurred accompanied by an increase in the red
blood cell fragility within 2 1/2 hours of injection. This anemia was
pronounced by 48-72 hours. In view of the severe lymphopenia and
neutrophilia which also occurred at 2 1/2 - 7 hours, they felt that
alloxan had caused an "alarm reaction". To them, this stressed the
toxic nature of alloxan.

Rose and György (68) similarly found that alloxan affected the
red blood cells of rats. On a tocopherol deficient diet, hemolysis of
up to 90% of the red blood cells occurred starting 10 minutes after the
alloxan injection. The severity of the hemolysis depended on the
degree of deficiency of tocopherol in the diet. Pre-treatment with
tocopherol prevented it. Other alloxan reduction compounds produced
the same result. Dialuric acid was even more effective. Cysteine
provided no protection. The phenomenon was apparently not related to
the reduction of hemoglobin by alloxan which also occurred. In vitro
experiments suggested that this activity of alloxan in blood might be
due to dialuric acid rather than alloxan or else another intermediate
compound.. This certainly indicates the complexity of the alloxan
reactions in blood.

Baiardi (5) also found hemolysis after alloxan although he did
not investigate this before 24 hours after injection.   This anemia
became more severe at 48 hours and it was a week before normal values
were approached.   He felt that his results did not indicate an "alarm
reaction" since the adrenals and spleen showed no histological change.
The initial decrease was due to the destruction or increased fragility
of the red blood cells and the later sustained anemia due to the effect
of alloxan on the erythropoietic cells of the bone marrow.   This
again suggests that the alloxan action involves tissue other than the
beta cells.

These results illustrate the rapid-acting, toxic nature of allo-
xan.   They do not explain the RR increase after alloxan injection, but
they make its appearance less unexpected.   The RR increase may even
be a result of some of these reactions.

The results also indicate how widespread is the reaction to
alloxan.   Besides the beta cells, the kidney, liver (50) and exo-
crine pancreas (57) are damaged.   Cataracts and retinal lesions may
also develop, in a much shorter time than in human diabetics (50).
In pigeons, purine metabolism is disrupted by alloxan.   Sodium urate
was found by Goldner and Gomori (34) covering the serous membranes
and infiltrating the kidney tubules of the birds a few days after
alloxan treatment.   Hyperglycemia was not produced, but elevated
blood uric levels were.   Similar results have been reported by Scott,
Harris, and Chen (72).   Besides these effects, alloxan has been reported
to inhibit numerous enzyme systems.   These few examples illustrate how

many different effects are produced by alloxan.

The view that, "Alloxan appears to be selectively toxic to the beta-cells" (45) is no longer tenable. Unfortunately it appears to be quite common. Hall, for example claims that "By means of alloxan injection, a form of experimental diabetes can be produced without any other side effects, such as those which follow removal of the pancreas, and without the necessity of an abdominal operation." (37). This assumption appears to be made by various people using tissue from alloxan-treated animals for enzyme studies (62). Alloxan, however, produces a number of side effects as well as hyperglycemia. This must be considered in such studies.

Alloxan and human diabetes are not the same thing. Both exhibit hyperglycemia, but this probably has not the same origin. Many human diabetics have apparently normal beta cells (30). Hyperglycemia in humans is only a symptom of a more fundamental disorder. Retinopathy, glomerulosclerosis, gangrene, symptoms of vascular disease are common in diabetics even when the glucose level is controlled by insulin (30). For these practical reasons as well as academic ones, increased understanding of diabetes is required.

Even though alloxan and human diabetes are not the same, alloxan is still used to study diabetes. Understanding of its action and the resulting effects is very important and it is hoped that this investigation can contribute to this. In summary, its purpose is to elucidate the nature and role of the rest reduction in blood before and after alloxan treatment and to add to the information about alloxan diabetes.

Materials and Methods

Toadfish

The first part of this investigation was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts, using the marine toadfish _Opsanus_ _tau_ (L.). The fish were obtained in small numbers sufficient for each experiment and were kept in wood and glass tanks with running sea water. Before use, they were weighed and tagged and allowed to recover at least 24 hours before use. Sixty-eight fish were used.

Blood was removed from the fish with an oxalated syringe (#23 needle) by way of the gill arch /60/. Alloxan (Eastman Kodak Lot #46) was dissolved immediately before use in iced distilled water, at a concentration of 14.7% (weight/volume). The usual dose level was 700 mgm/km /60/. Doses of 300, 450, and 600 mgm/km were also used. A few fish were injected with more dilute solutions of alloxan. Control fish were injected with iced distilled water. All injections were made by direct heart puncture through the ventral body wall /60/.

For all blood samples, duplicate determinations were made for blood sugar by Murrell's modification of Folin's procedure and the glucose oxidase procedure of Saifer and Gerstenfeld (see Appendix). The following number of determinations were made: normal blood - 57; alloxan-treated blood - 43 (at different times after injection); control - 7. Repeated sampling could not be carried out on the same fish as anemia would result.

After the last sampling, the fish were killed and pancreas $\overline{/60/}$, kidney, liver, and gills were removed and fixed immediately.

Catfish

Catfish, _Ictalurus nebulosus_ (La Sueur), were used at McMaster University.   They were obtained from the Port Rowan Fisherman's Co-operative, Lake Erie, in groups in the spring and fall and held in a large glass aquarium supplied with running spring water and an air supply.   The fish were weighed and tagged and allowed 24 hours to re-cover before use.   Sixty-two fish were used.

Blood was withdrawn for blood sugar determinations by means of a heparinized syringe from the gill arch, the heart (through the ventral surface), and the conus arteriosus /55/.   For chromatography, 0.5 ml of blood was withdrawn at the same time.   Alloxan (lot #46) was made up as before at a concentration of 10% (weight/volume) and injected at a dose level of 400 mgm/km body weight /55/, through the conus arteriosus. Corresponding control injections were of iced distilled water.   Experi-mental and control fish were used in pairs, usually paired by weight.

Duplicate determinations of blood sugars were made.   For normal blood sugar, 46 determinations were made and 79 for alloxan and control fish at times from 15 minutes to 24 hours after injection.

At the end of the last sampling, the fish were killed.   Pancreas /55/, kidney, liver, head kidney and gills were removed and fixed immediately.

Other Colorimetric Determinations

Uric acid was determined at 0 and 1 hours for 4 alloxan and one control catfish, by the Folin direct and indirect methods (as outlined in Lewis and Christman /48/).

Glucuronic acid was determined by the naphthoresorcinol method of Bray /14/ in 2 alloxan-treated catfish at 0 and 1 hours. By the method of Ratish and Bullowa /67/ 4 fish were used for glucuronic acid determinations at 0 and 3.5 hours. The method of Dische /21/ using a carbazole reagent was discarded immediately because the reagent reacted with tungstic acid to produce a very dark colour.

Tissue Processing

All tissues were fixed in Bouins fluid, dehydrated in cellosolve, cleared in benzene, and embedded in Tissuemat (60-63°C). Only a few gills were sectioned. These were cut at 10 µ and stained with haematoxylin and triosin and periodic-acid Schiff stains.

Chromatography

A description of the apparatus, paper, solvents, indicators, and technique used will be found in the Appendix.

A number of preliminary runs were made. The results of these are included only to extend the number of R_f values and to show the results of the different solvents. The methods used are indicated where the results are given.

Blood from one group of catfish (4 alloxan, 1 control) was treated with pyridine (see Appendix). Samples were 0 hr. - 5, 1 hr - 5, 3 hr. - 1, 6 hr. - 1, 9 hr. - 1, 24 hours - 3.

Blood from the second group of catfish (3 alloxan, 3 controls) was treated with alcohol (see Appendix). Samples were taken at 0 hr. and one hour only.

The solvents used most frequently were i-propanol, pyridine, acetic acid, water (PPAW); butanol, pyridine, water (BPW); benzene, butanol, pyridine, water (BBPW); propanol, ethyl acetate, water (PEAW); butanol, ethanol, water, acetone (BEWA). PPAW gave the best separation. The papers were run in two different solvents at right angles (2-way) or twice in the same solvent the same way (2X). (See Appendix.)

The chromatograms were developed in alkaline silver nitrate, Glucostat reagent, and ninhydrin (see Appendix). When dry, they were photographed. The spots were marked and they were rephotographed. The distances the spots had moved were measured and R_f values calculated.

Results

Toadfish Blood Sugar

The results of the glucose oxidase and Folin determinations
are presented in Table I.   The numbers there represent the averaged
values.   The figures in brackets indicate the number of determina-
tions.   The third column, the Folin value less the glucose oxidase
value, is taken to represent the rest reduction.   In 9 of the 57
fish, the normal glucose oxidase value was higher than the Folin value.
These figures were not averaged with the rest.   These results are
plotted in Figure 1.

The average normal blood sugar value was 30 mgm % (range 2-60)
by the Folin method, 19 mgm % (0-50) by the glucose oxidase procedure,
and the RR was 17 mgm % (0-35).

The results indicate that after alloxan injection a definite
increase in the RR occurred beginning at 10 minutes which reached a peak
at one hour.   By 3 hours, the level of RR had begun to drop and at
6 and 9 hours appeared below normal.   From the few values obtained
after this, it is difficult to tell when the normal level was reached.
It is interesting when comparing the true glucose and the RR curves
(Fig. 1) to note that the RR level dropped as the true glucose level
rose.

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TABLE I

Blood Sugar Determinations in Toadfish

Time	Alloxan Treated			Control		
	Folin	Gl.Ox.	Folin-Gl.Ox.	Folin	Gl.Ox.	Folin-Gl.Ox.
0 hr	30 (57)	19	17 (46)			
10 min	68 (1)	37	31			
30 min	69 (2)	38	31			
45 min	57 (2)	10	47			
1 hr	126 (6)	23	103	59 (2)	.5	58
3 hr	87 (12)	32	55	40 (6)	26	14
5 hr	79 (3)	32	46			
6 hr	79 (4)	70	10			
9 hr	152 (2)	150	2	42 (1)	47	-5
12 hr	50 (1)	54	-4			
24 hr	117 (7)	97	28			
48 hr	61 (2)	66	28 (1)			
5 days	66 (1)	50	16			

Values in mgm/100 ml blood

Number of determinations in brackets

FIGURE 1. TOADFISH REDUCING SUBSTANCES IN ALLOXAN BLOOD
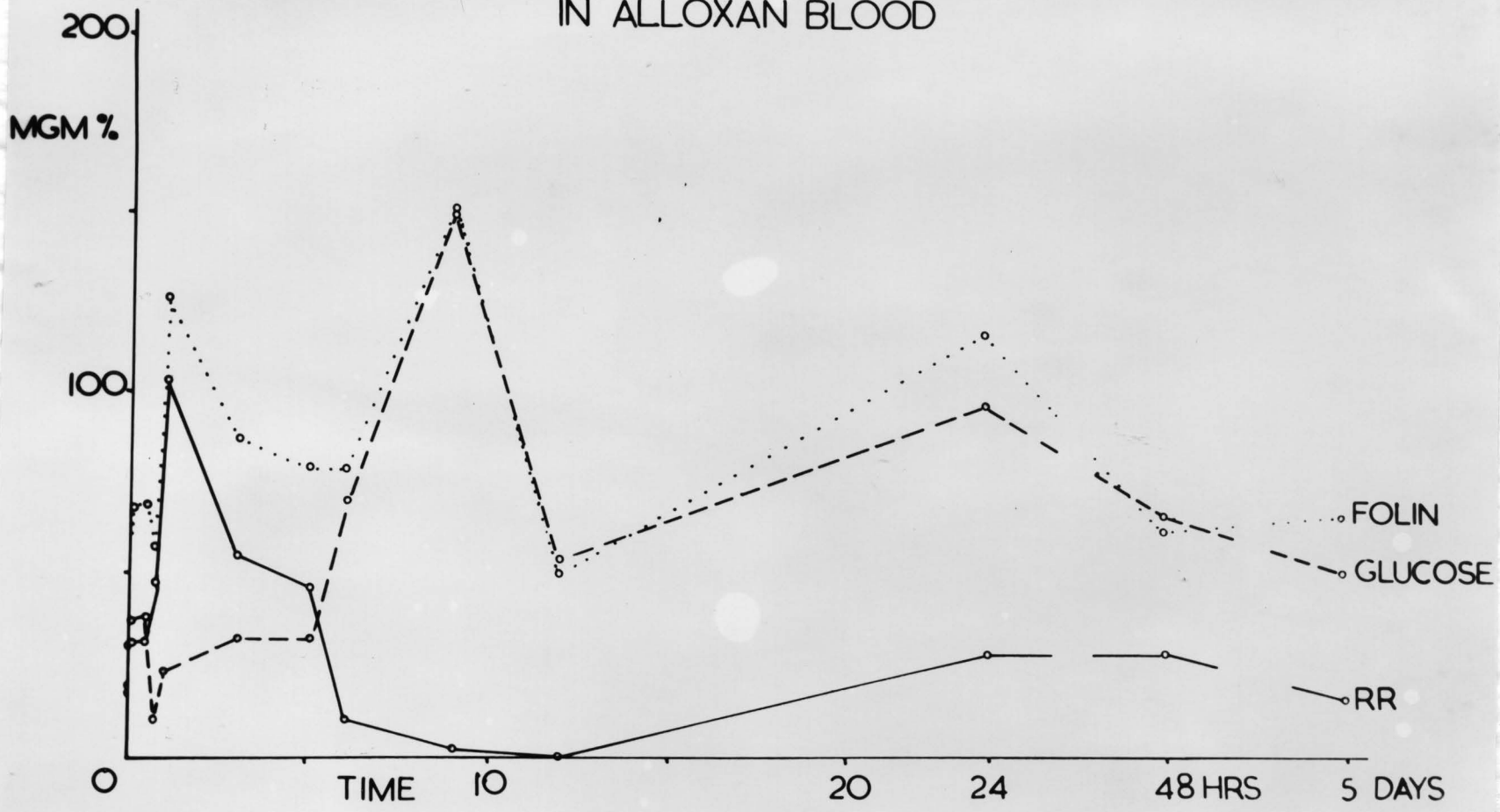
Considerable variation in the response to alloxan was found. Some fish appeared to develop very high levels of RR, while others did not. For example at one hour, 2 fish had RR values of more than 180 mgm %, 2 had values of about 100mgm %, and the other 2, values of around 20 mgm %, quite close to normal.

The one fish injected with alloxan at 450 mgm/km instead of 700 mgm/km showed 152 mgm % RR at three hours. Likewise those injected with 600 mgm/km did not appear to differ. However, the 3 fish injected with 300 mgm/km in a more dilute solution did not exhibit a RR increased above normal.

Catfish Blood Sugar

The results of the glucose oxidase and Folin determinations are presented in Table II and plotted in Figures 2 and 3. The normal blood sugar values and their ranges were Folin 30 mgm % (5-88); glucose oxidase 22 mgm % (9-45); and RR 14 mgm % (0-56). Twelve of the 45 determinations for normal blood sugar resulted in glucose oxidase values higher than the Folin values. These were not averaged for the RR average value.

The results show a definite increase in the RR beginning at 15 minutes after alloxan injection. The peak at one hour was maintained in the catfish past three hours. At six hours, the RR level had begun to drop and by 24 hours appeared to be normal. The graph again indicates that the RR level dropped as the glucose level rose. No drop in RR to below normal values was observed here.

TABLE II

Blood Sugar Determinations in Catfish

Time	Alloxan Treated			Control		
	Folin	Gl.Ox.	Folin-Gl.Ox.	Folin	Gl.Ox.	Folin-Gl.Ox.
0	30 (45)	22	14 (33)			
15 min	69 (1)	40	29	50 (2)	26	24
30 min	70 (2)	17	53	54 (2)	35	19
1 hr	111 (19)	36	80 (18)	46 (13)	36 (12)	16 (8)
3 hr	112 (12)	40	72	66 (8)	35	31
6 hr	130 (4)	106	25	63 (3)	45	18
9 hr	49 (2)	18	31	63 (3)	38	30
24 hr	55 (2)	37	19	25 (1)	22	3

Values in mgm/100 ml blood

Number of determinations in brackets

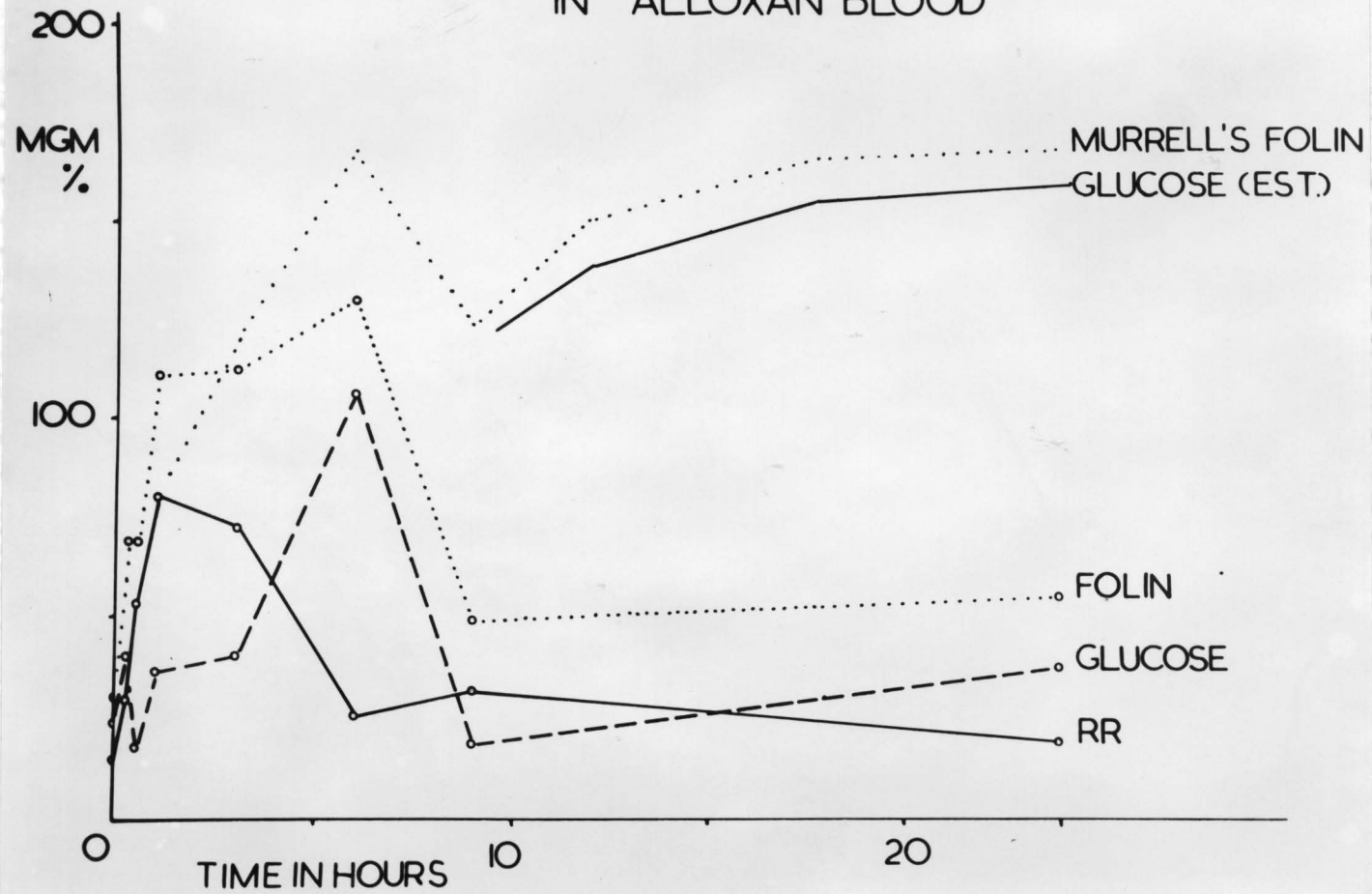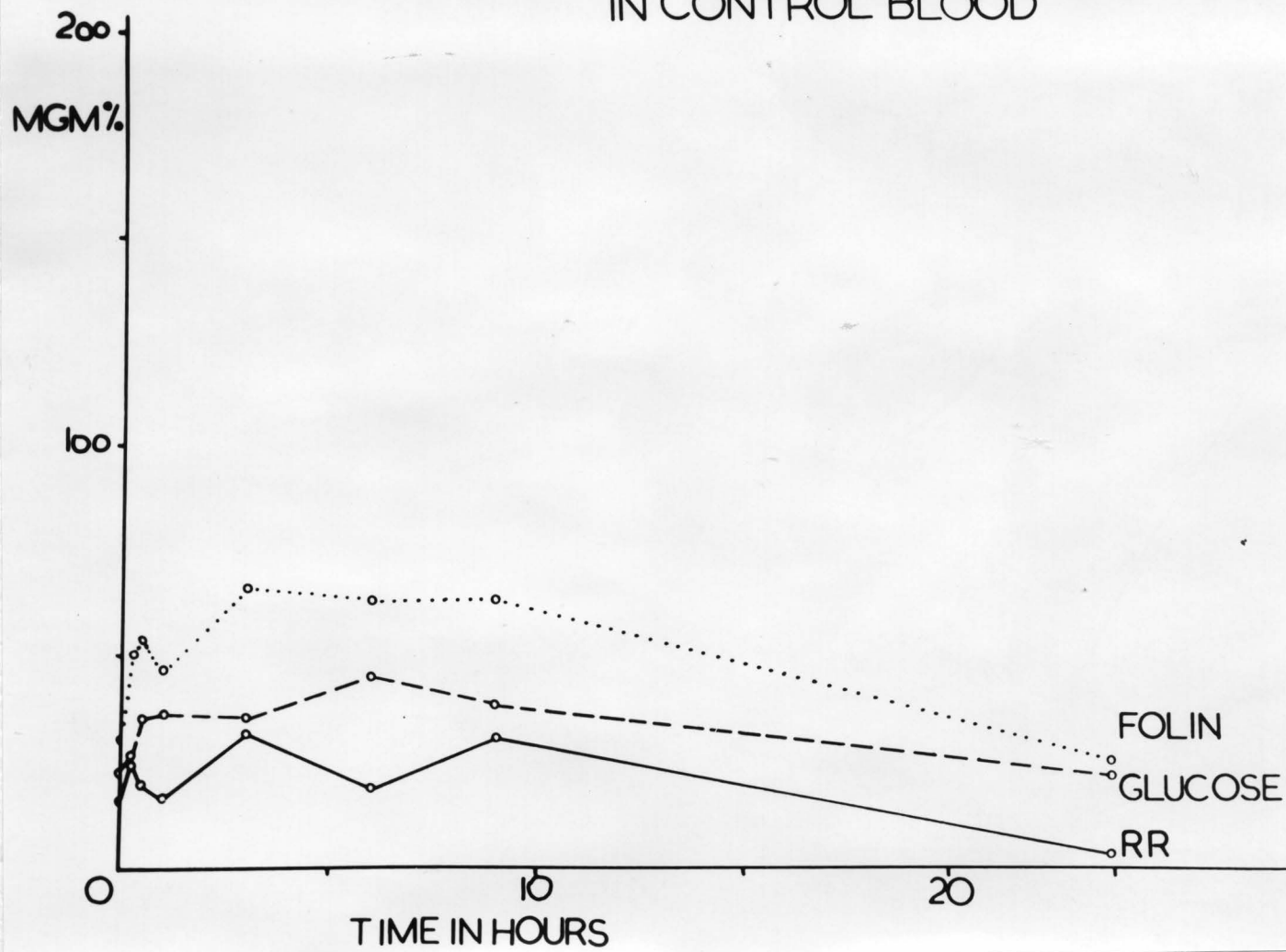FIGURE 2. CATFISH REDUCING SUBSTANCES IN ALLOXAN BLOOD

FIGURE 3. CATFISH REDUCING SUBSTANCES IN CONTROL BLOOD

MGM %

200

100

FOLIN

GLUCOSE

RR

0   10   20

TIME IN HOURS

21

A variation in response similar to that in toadfish was seen in the catfish. Of the 19 determinations at one hour, 5 of the RR values were about normal, while 7 were more than 100 mgm %.

Part of the curve obtained by Murrell (55) working with catfish under similar conditions is included in Fig. 2. The values found during this investigation were lower. This is probably due to the fact that a higher percentage of fish failed to respond to alloxan. A high mortality rate was found among the fish which did respond. These factors account for the decrease in glucose levels found at 9 hours, when Murrell found no such drop. This could be due to a number of factors such as alloxan lot variation and seasonal variation in the condition of the fish. An estimate of the probable glucose level for Murrell's results has been made.

Visual Observations

It should be mentioned that in many cases, the blood samples obtained after alloxan were darker and more brown than normal. This occurred most frequently in the blood of fish which showed a large RR. This is probably due to the reduction of hemoglobin by alloxan. Such fish very often exhibited very pale gills. No records of this were kept.

Other Colorimetric Procedures
Uric Acid

The averaged results of the determinations for uric acid for five normal fish were by the Folin direct method 0.11 mgm % and by the Folin indirect method 0.29 mgm %. At one hour, the four alloxan treated

fish showed values of 0.32 mgm % by the Folin direct and 0.09 mgm % by the Folin indirect.   The one control fish showed uric acid levels of 0.15 mgm % and 0.9 mgm % respectively.

The Folin indirect method is considered to be the more specific method (48).   These results indicated that uric acid was not involved and no further determinations were made.

Glucuronic Acid

By the naphthoresorcinol method of Bray the value for glucuronic acid in normal blood was 78 for 2 fish and 70 one hour after alloxan injection.   Proper standards were not set up, and the numbers given are not themselves significant.   The comparison, however, is.

By the naphthoresorcinol method of Ratish and Bullowa, the value for glucuronic acid in normal fish blood was 25 (3 determinations) and 3 1/2 hours after alloxan for 2 fish was 30.   In both cases significant increases in the RR occurred.

These results again are not the result of a thorough investigation.   They indicated that this approach to the problem was not likely to be fruitful.

Histological Results

Gills were examined from 7 alloxan-treated, 1 control, and 2 normal toadfish.   The fish were killed at 1, 3, and 36 hours after alloxan injection.   In 6 of the 7 alloxan-treated fish, the epithelial layer of the gill filaments was completely separated away from the basement membrane between it and the capillary.   (See photographs). (Magnification 375X).
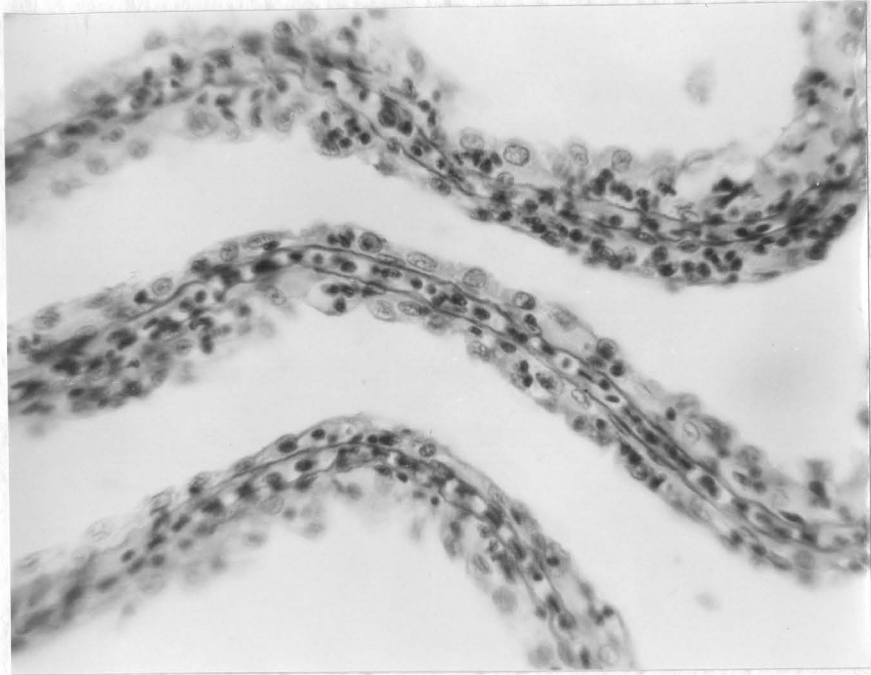
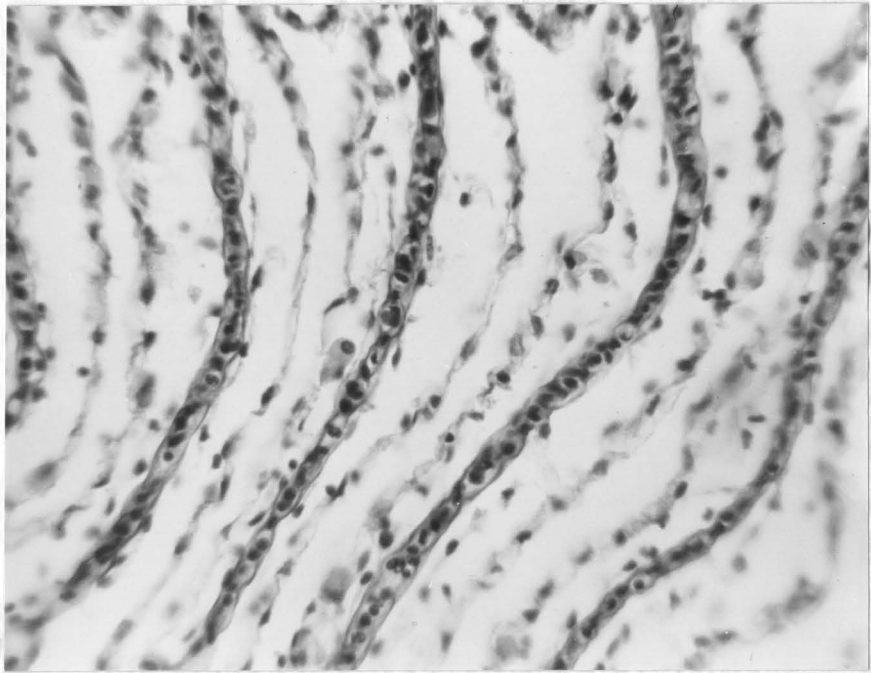Plate 1.   Normal Toadfish gill

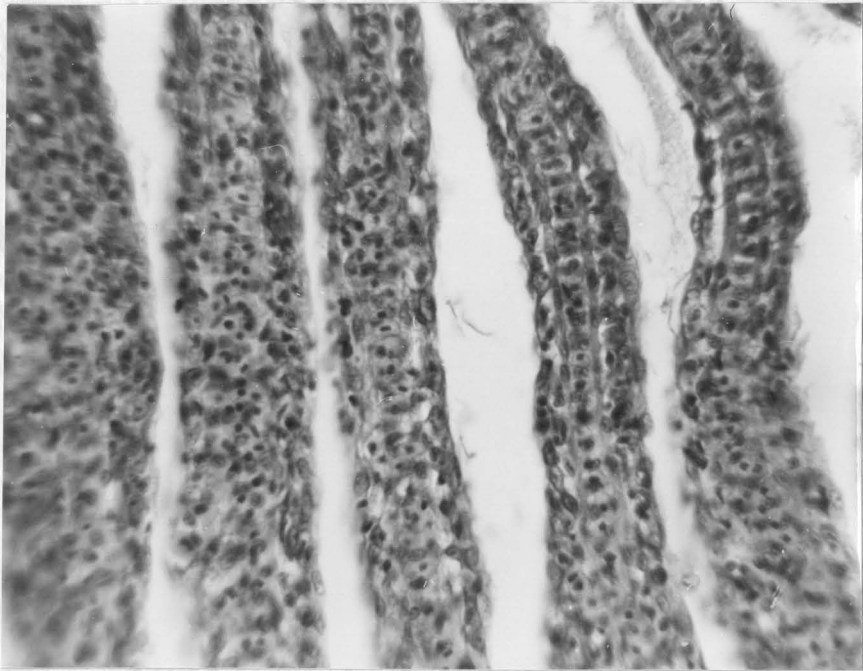Plate 2.   Toadfish gill, 3 hours after alloxan
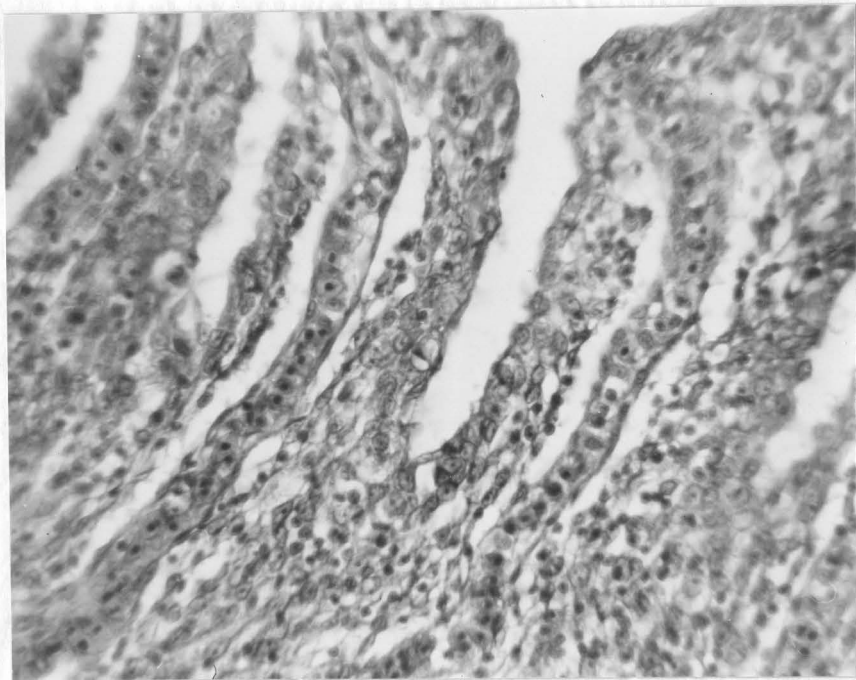
Plate 3.   Normal catfish gill

Plate 4.   Catfish gill, 3 hours after alloxan

This did not occur in the normal and control fish.

Gills were examined from 6 alloxan-treated and 4 control catfish. The fish were killed 3 hours after injection. The gill structure of catfish is much different from that of toadfish and makes the detection of this phenomenon difficult. It may have occurred in 4 of the 6, but it is not certain.

This examination was carried out because the paleness of the gills suggested that some pathological changes might be apparent. It is not thorough and more work should be done.

Chromatography Results

The R_f values for glucose in 13 different solvents are given in Table III, for the convenience of anyone else doing similar work. They indicate the superiority of butanol, pyridine, water (BPW) and i-propanol, pyridine, acetic acid, water (PPAW) as solvents.

The results of the chromatograms run in these solvents are given in Tables IV and V. They show the R_f values of glucose and other unidentified compounds run under different conditions (see Appendix). The compound X is possibly either glucuronic acid (glucuronide) or glutathione. This compound was nearly always present and so has been separated from the other unknowns. It was usually present in concentrations similar to glucose (i.e. the spots were about same size and colour) and both were usually much clearer on the paper than anything else. Its R_f value is similar to that of glucuronic acid samples run at the same time (see Plates 11 and 12), but is also similar to that of several prominent ninhydrin positive spots. Its identity will be determined later.

TABLE III

Glucose R_f Values

Solvent	Glucose R_f Values	
	#1 Paper	#20 Paper
i-propanol, pyridine, acetic acid, water	56 (10)	51 (18)
butanol, pyridine, water	46 (5)	43 (23)
butanol, ethanol, water, acetone	31 (7)	22 (9)
benzene, butanol, pyridine, water	33 (16)	25 (5)
phenol, water	35 (5)	
butanol, ethanol, water, NH_4OH	53 (1)	
butanol, ethanol, water	12 (1)	
amyl alcohol, pyridine, water	31 (1)	
n-propanol, ethyl acetate, water	42 (8)	
collidine, lutidine, water	53 (1)	
butanol, acetic acid, water	26 (1)	
butanol, water	0.1 (1)	
ethyl acetate, acetic acid, water	24 (1)	

R_f value = $\dfrac{\text{distance spot moved}}{\text{distance front moved}}$ x 100

Number of determinations in brackets

TABLE IV

R_f Values for Glucose and Unknown Compounds Separated by Butanol, Pyridine, Water (BPW)

P	Run	T	Glucose R_f	X R_f	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-100
1		N	46 (2)	28 (1)										
		A'	45 (3)	20 (2)							64 (1)			
20		N	43 (7)	26 (7)		11 (1)		32 (3)	47 (1)			77 (1)		93 (1)
		A'	43 (4)	23 (4)			25 (2)*		49 (1)		68 (1)			95 (2)
		C'	44 (6)	25 (6)				50 (1)* 33 (1)						92 (2)
20	2nd w	N	44 (5)	24 (5)										
		A'	45 (2)	26 (2)		13 (1)	29 (1) 22 (1) 25 (1)	31 (1)	44 (1)		64 (1)			
		C'	45 (2)	24 (2)				39 (1) 31 (1)						
20	2X	N	59 (12)	34 (12)				36 (12)	45 (6)*	51 (2)				95 (12)
		A'	57 (7)	32 (7)			26 (2)*	32 (2)	49 (5)*	50 (1)		72 (1)	83 (1)	94 (5)
		C'	61 (7)	37 (6)						50 (3)*				95 (6)

Abbreviations $R_f = \dfrac{\text{distance spot moved}}{\text{distance front moved}} \times 100$

P = paper type

T = treatment N - normal blood
A' - one hour after alloxan injection
A^3 - three "   "   "   "
C' - control one hour after d.w. injection

Run - manner of running solvent (see Appendix)

Numbers in brackets represent number of chromatograms spot found

Values arranged according to ranges for convenience

* Oxidizing Spots

TABLE V

R_f Values for Glucose and Unknown Compounds Separated by i-Propanol, Pyridine, Acetic Acid, Water (PPAW)

P	Run	T	Glucose R_f	X	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-100
1		N	52 (2)	24 (1)										
		A'	58 (5)	25 (4)	5 (1)							76 (2)		
		A3	47 (1)	24 (1)	11 (1)			37 (1)						
		A6	54 (1)	16 (1)					41 (1)			74 (1)		
		A24	57 (1)				24 (1)					77 (1)		
1	2nd w	N	56 (5)	26 (4)			21 (1)	38 (1)						
		A'	44 (1)	9 (1)	.7 (1)			31 (1)			60 (1)			
		A3	58 (3)	28 (2)	.8 (1)			39 (1)	48 (1)			70 (1)		
		C'	66 (1)	16 (1)	.7 (1)		29 (1)							
		C24	63 (1)	28 (1)										
1	2X	N	79 (4)	40 (3)				35 (2)	41 (3)					
		A'	81 (2)	43 (3)							60 (1)			96 (1)
		C'	79 (2)	41 (2)				35 (1)	40 (1)					97 (1)
20		N	49 (7)	20 (7)			27 (2)*	37 (1)*	42 (1)*				87 (1)	97 (1)
		A'	50 (4)	20 (1)		12 (1) / 17 (1)	24 (3) / 22 (1)	35 (1) / 39 (1)*	42 (1)		65 (1)	75 (2)	85 (1)	
		C'	51 (3)	21 (5)			23 (1)	32 (1)						
20	2nd w	N	54 (5)	23 (5)				39 (1)	42 (1)	57 (1)		72 (1)		
		A'	55 (2)	24 (2)					41 (1)					
		C'	54 (3)	23 (3)			21 (1)	33 (1) / 39 (1)						
20	2X	N	71 (11)	34 (10)			20 (3) / 23 (1) / 29 (1)	37 (2)	43 (2)*		60 (5)			94 (8)
		A'	71 (7)	34 (7)			22 (2) / 28 (1)	37 (3)*	44 (3)*	54 (2)	61 (3)		86 (2)*	93 (5)
		C'	73 (6)	35 (5)			20 (2) / 25 (1)	31 (1) / 37 (1)						95 (5)

In normal blood, glucose was always found. If it was not, then the sample concentration was assumed to be too low. In 76 cases with 5 different solvents, the compound X appeared in 66. This is the dark spot below glucose in Plate 7. It was present in a concentration only slightly less than that of glucose. In nearly half of the blood samples treated with alcohol (and in none of those treated with pyridine) another compound was found with a very high R_f value. (See Plates 13-16).

Besides these 3 reducing spots, several others appeared in much lower concentrations and only occasionally (in approximately 10% of the samples). In about 25% of the samples (mainly those alcohol treated) oxidizing spot(s) are found between glucose and X (see Plate 13). These are referred to as oxidizing because they are white whereas the rest of the paper background is grey. There did not appear to be a very definite pattern in the spots. However, glucose, X, and possibly the high R_f compound were usually present, while oxidizing compounds and small amounts of other compounds were present sometimes.

In the blood of fish injected with distilled water, the results were very similar. Of 33 samples, 30 contained X. The high R_f value compound appeared in 12 cases, all alcohol treated. Four showed oxidizing spots. Various other compounds appeared in about the same proportion of samples as normal blood. However Plates 13 and 14 do indicate clearly that the blood of fish one hour after distilled water injection was not exactly the same as it was before. Both show a slight increase in glucose and a change in X and in the material with

R_f values between X and glucose, particularly a decrease in the oxidizing material.

In the blood of alloxan-injected fish, the results were not so clear. Glucose was usually, but not always, present in larger amounts than before injection. X was frequently present in slightly lower concentration but not always. The high R_f value compound appeared to be unaffected. Only one spot appeared consistently in alloxan blood. It has an R_f value half as high again as glucose (see Plates 8, 9, 10) and appeared more often in the pyridine-treated samples than in the alcohol-treated ones (see Plates 13 and 15). Again noticeable differences were seen between this and normal blood, especially if the RR was large. Most striking is the lack of reducing material.

The results of dipping the chromatogram in ninhydrin were very interesting. In a number of cases, the number and concentration of amino acid compounds was greatly reduced after alloxan injection. This did not always occur to the extent it did in Plate 18. Sometimes there was little difference. No attempt was made to identify any of the compounds.

The difficulties involved in making these determinations were considerable. Each blood sample used contained only 0.5 mgm RR (mgm glucose equivalents) and 0.1 mgm glucose if the RR was 100 mgm % and the glucose 20 mgm %. In the processing some of this undoubtedly was lost, so that only very small amounts were available for chromatography. Only a few chromatograms for each sample could be run. The work was limited by material and also by time, and had to be ended. The results are thus incomplete.

Explanation of Chromatogram Plates

Plate 5   Key to Plates 7-10

Plate 6   "   "   "   11-17

Plate #	Sample Lft. to Right	Blood Sugar F-M	GO	RR	TR. Treatment	Solvents				Developer
7	#71 Normal	13	9	4	Pyridine	BBPW BEWA		20	31cm^2	ASN silver nitrate
8	#71 A'	128	16	112	"	"	"	20	"	"
9	#68 A'	57	34	23	"	BBPW BEWA		20	"	"
10	#69 A'	155	34	121	"	BBPW BEWA		1	"	"
11	#87 Normal	34	44	-10	Alcohol	PPAW		20	"	"
	Stds glucose gluc acid				"	"				"
	#87 Normal		"		"	PPAW 2X				"
	Std.					"				"
12	#87 A'	136	31	105	Alcohol	PPAW		20	"	ASN
	Stds					"				
	#87 A'					PPAW 2X				"
	Stds					"				
13	84 N	11	31	-20	Alcohol	BPW 2X		20	"	ASN
	84 A'	87	34	53		"				
	88 N	17	19	- 2		"				
	88 C'	40	42	- 2		"				
14	84 N				Alcohol	PPAW 2X		20	"	ASN
	84 A'		"			"				
	88 N					"				
	88 C'					"				

Plate #	Sample Lft. to Right		Blood Sugar F-M	GO	RR	TR. Treatment	Solvents			Developer
15	92	N	9	24	-13	Alcohol	PPAW 2X	20	31cm^2	ASN
	92	A'	162	47	115					
	94	N	46	37	9					
	94	C'	59	61	- 2					
16	92	N								
	92	A'		"		"	BPW 2X	20	"	ASN
	94	N								
	94	C'								
17	92	N		"		"	PPAW 2X	1	41 cm	ASN
	92	A'								
	94	N								
	94	C'								
18	92	N		"		"	PPAW 2X	1	41 cm long	NINHYDRIN
	92	A'								
	94	N								
	94	C'								
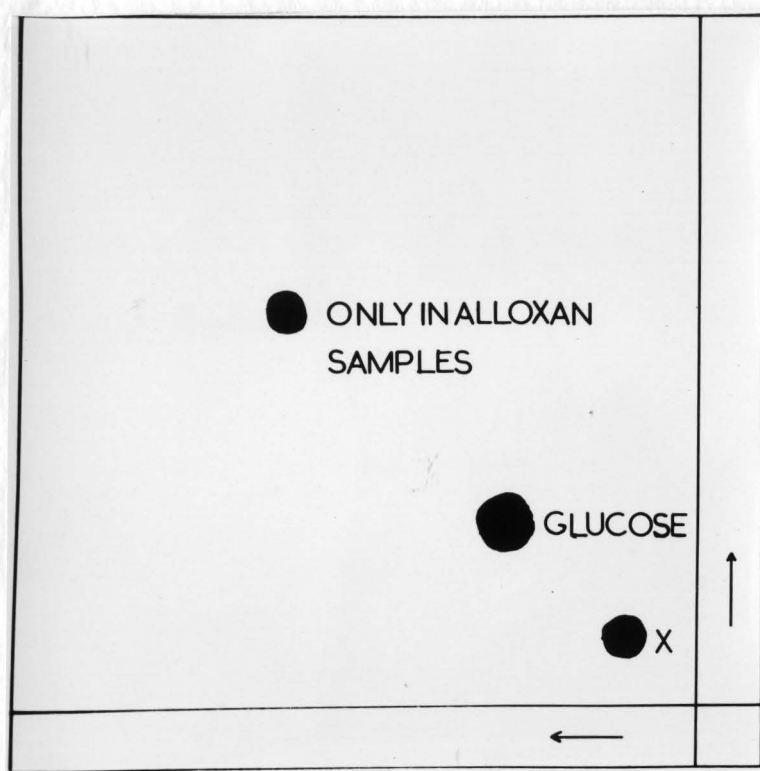
Plate 5. Key to Plates 7-10

Plate 6. Key to Plates 11-17
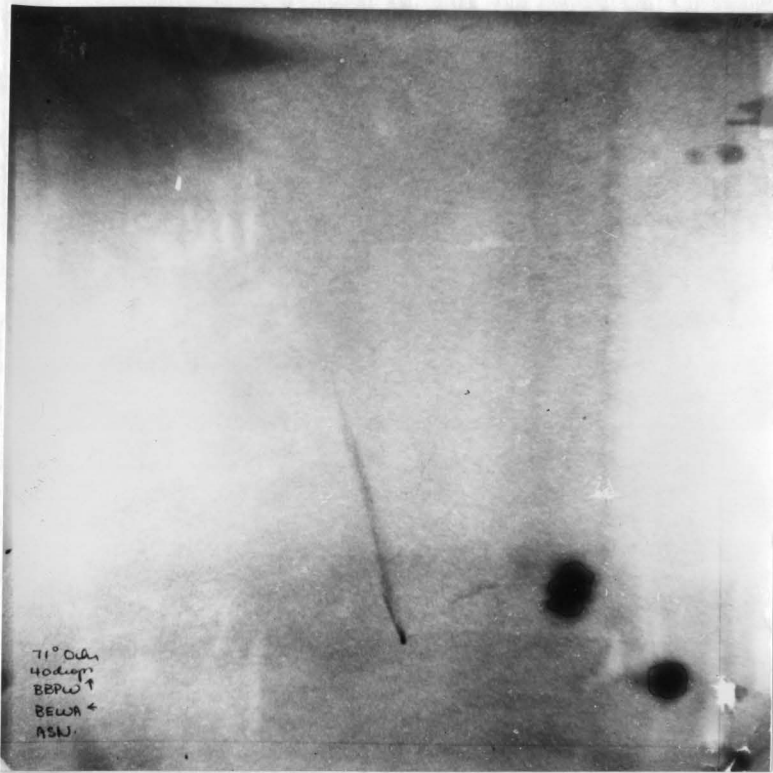
Plate 7.   Fish #71.   Normal Blood
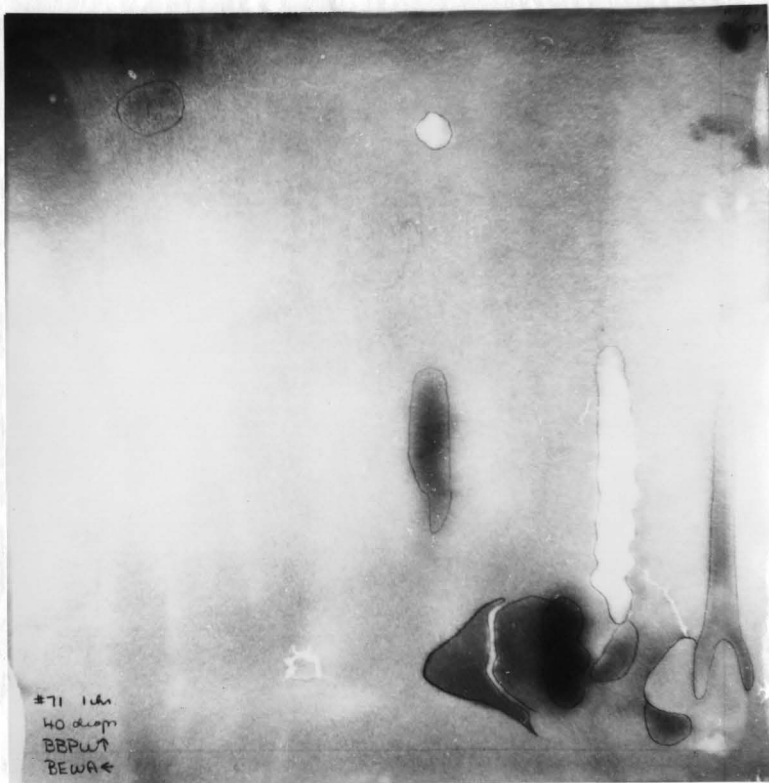
Plate 8.   Fish #71.   One hour after alloxan.
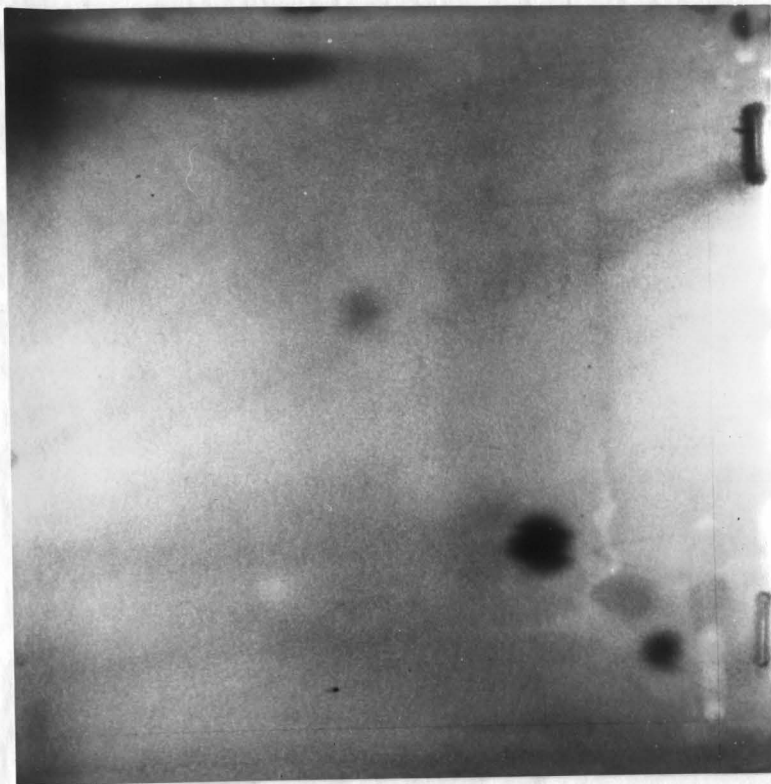
Plate 9.   #68.   One hour after alloxan

Plate 10.   #69.   One hour after alloxan
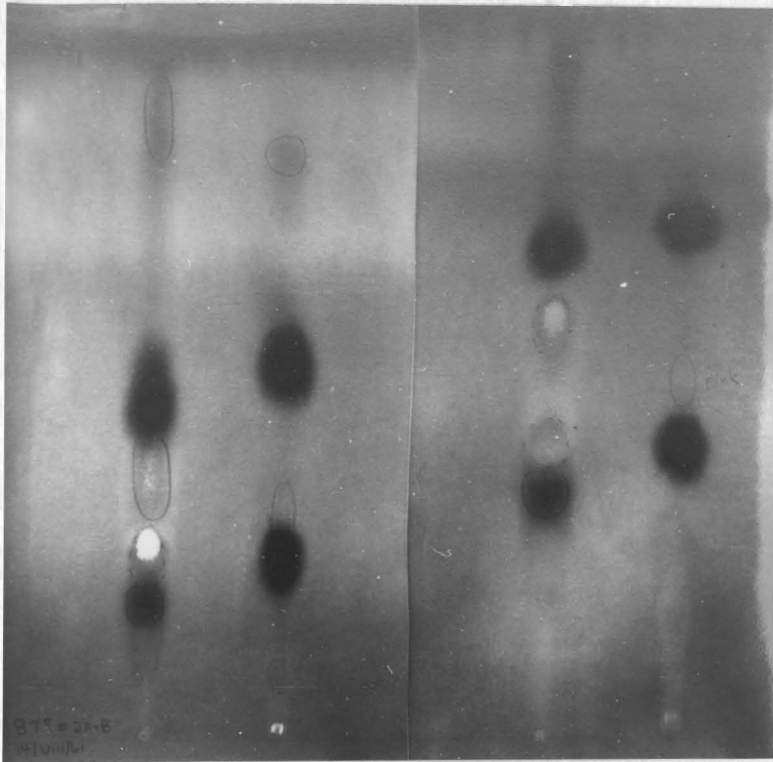
Plate 11.   #87.   Normal and Standards
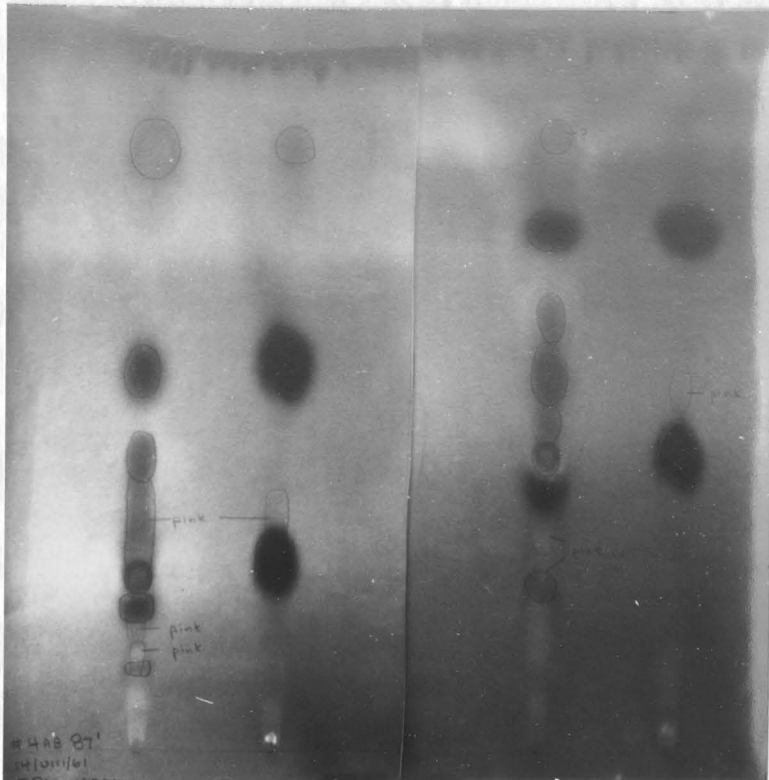
Plate 12.   #87.   One hour after alloxan and Standards

36
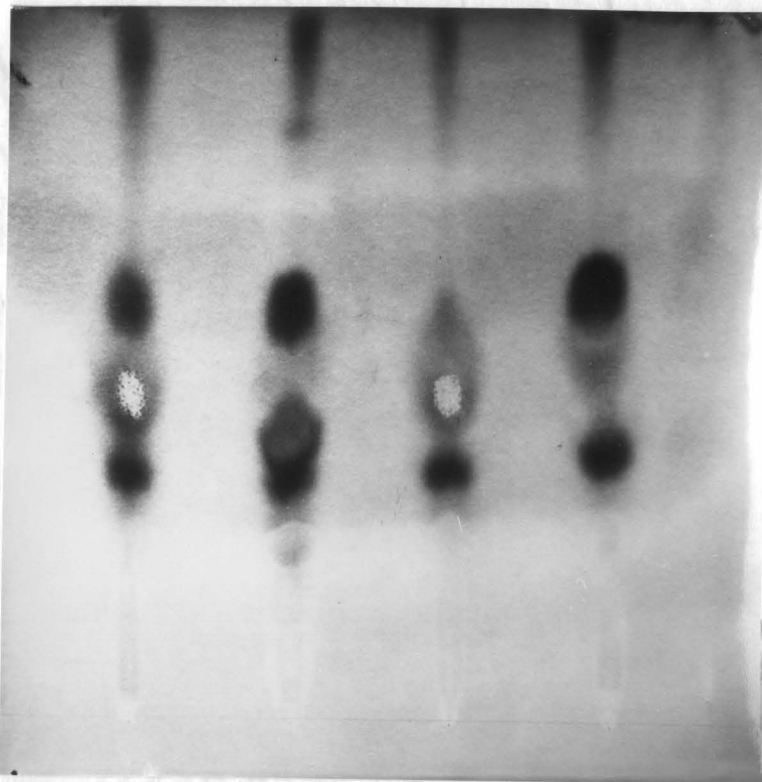
Plate 13.   #84 Normal and Alloxan
             #88 Normal and Control
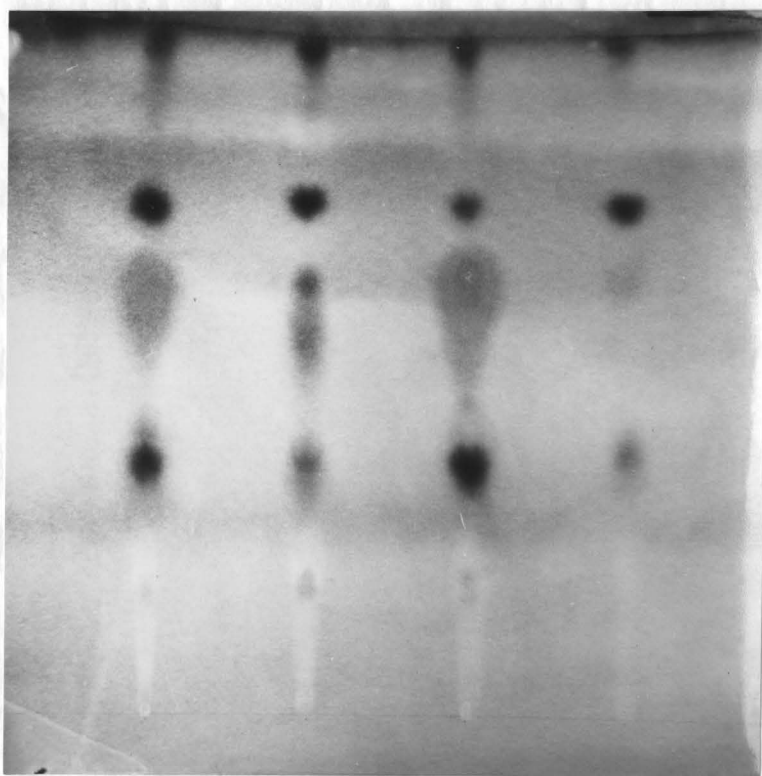
Plate 14.   #84 Normal and Alloxan
             #88 Normal and Control
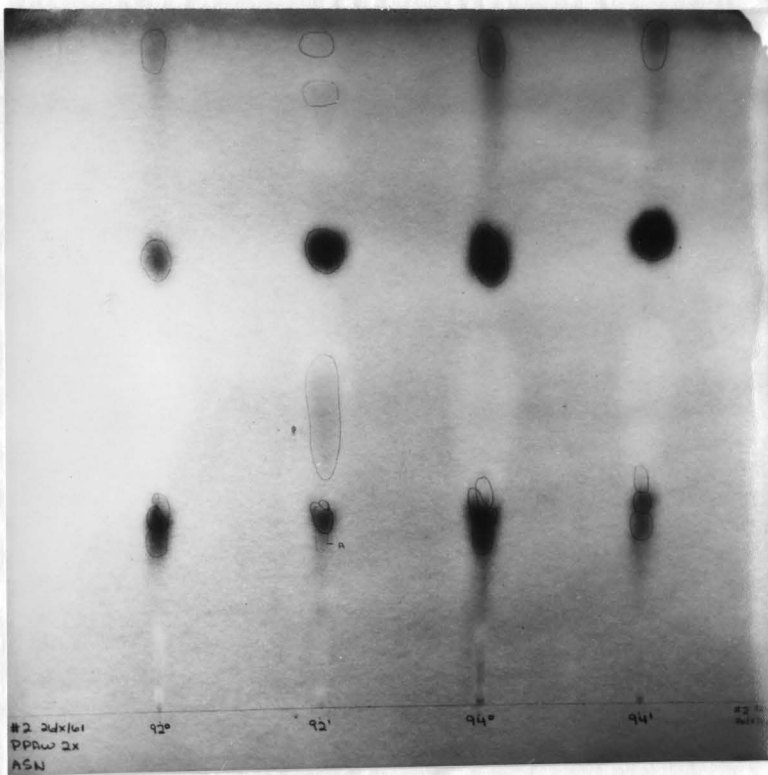
Plate 15.　#92 Normal and Alloxan
　　　　　　#94 Normal and Control
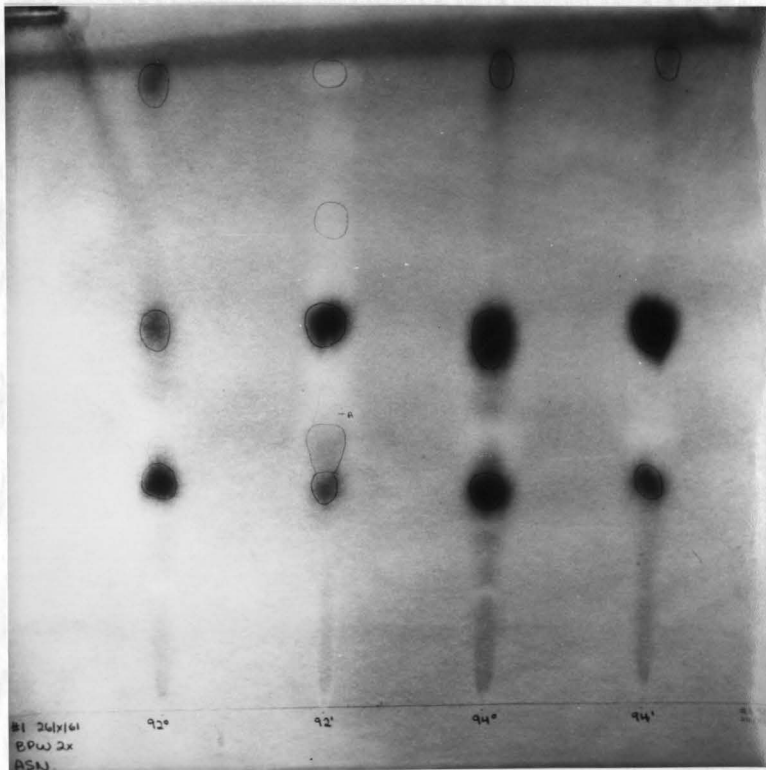
Plate 16.　#92 Normal and Alloxan
　　　　　　#94 Normal and Control
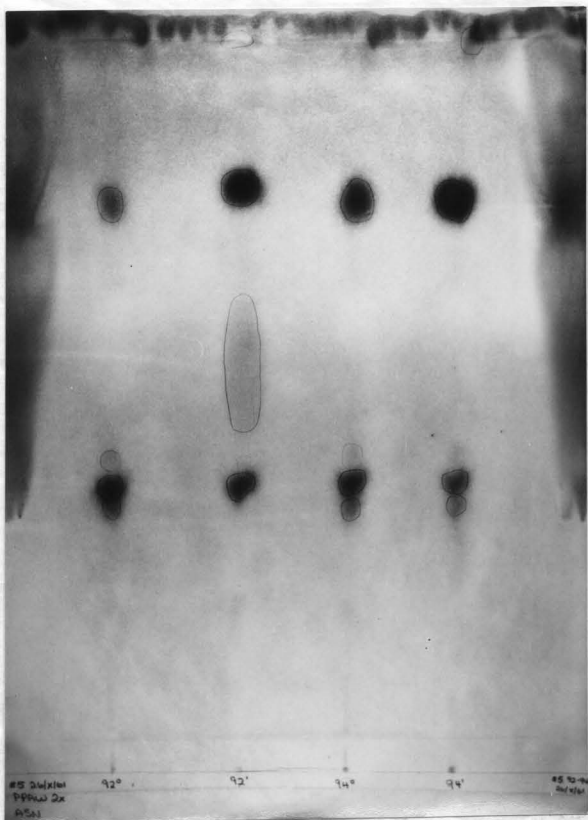
Plate 17.   #92 Normal and Alloxan
            #94 Normal and Control
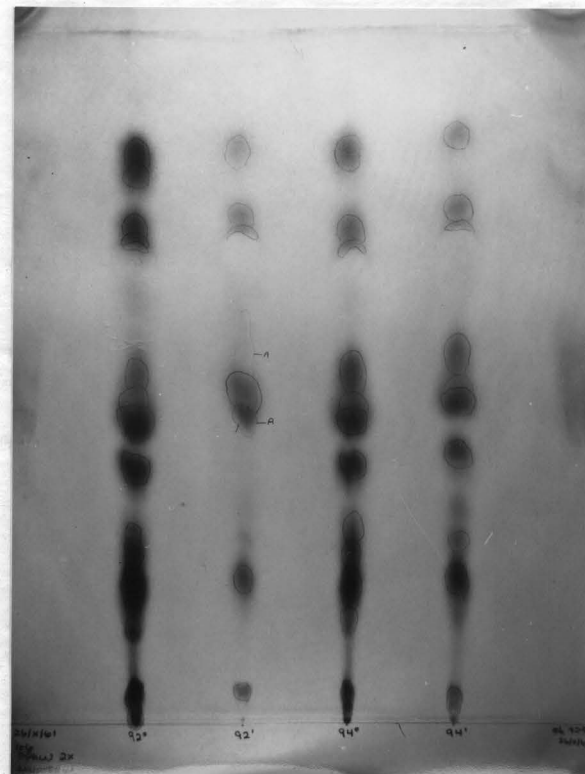
Plate 18.   #92 Normal and Alloxan
            #94 Normal and Control

            Ninhydrin

Discussion

The results of this investigation indicate the complexity of the problem. Although most of the questions asked originally have not been answered, considerable information has resulted. The RR material has not been identified but more accurate speculation about it is now possible. As a result, a number of new approaches to the problem can be suggested. More research is required and provides an interesting challenge.

Only one of the original questions asked is answered. This is the question of when the RR appears and for how long it is present. This was shown by the blood sugar determinations. The RR began to increase within fifteen minutes of the alloxan injection reaching a maximum one hour later. At this time the amount of RR (measured in mgm % glucose equivalents) present was more than twice that of glucose, while normally there was less. The level of RR by three hours had begun to decrease slowly and by twenty-four hours was normal. The level of glucose rose more slowly and too few values were determined at the later periods to follow its course. This generally represents the results of the RR and glucose determinations in both the toadfish and catfish.

The validity of these results depends on the validity of the methods used. That the Folin method measures reducing substances rather than glucose has long been known. Murrell found it to be accurate and

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reproducible in the range 15-200 mgm % glucose. For 200 duplicate determinations he found an average deviation of 3 mgm % (55). The increase in reducing substances may then be accepted as a true one.

The next question is whether one can be sure this increase is not due to glucose. How accurate is the glucose oxidase procedure? The enzyme preparation appears to act very slowly on mannose, galactose, and xylose (31, 40). Under the condition of the procedure used, this is not likely to cause an appreciable affect. A more serious error can result from inhibition of the reaction. Any other substrate of peroxidase such as uric acid, ascorbic acid, bilirubin and catecholamines may competitively inhibit the reaction, if present in large amounts (81). Falkmer measured some of these in normal blood and found them present in only very small amounts (25). It seems unlikely that any of these substances could be produced rapidly in amounts large enough to produce inhibition since excess enzyme is present. Although the possibility still exists that the glucose oxidase values are too low, it is not unreasonable to accept them as fairly accurate. That changes in the RR have been measured by other procedures supports the view that it was the RR, not glucose, which increased.

The results of the uric acid determinations while not complete suggest that uric acid is not a major part of the RR. In normal fish blood the level of uric acid is 2.6 mgm % in carp, 8.6 mgm % in trout (22), and 0.51-0.86 mgm % in sculpin (25). The values found here were similarly low and did not show a real increase after alloxan. Thus it does not seem likely that uric acid could account for the alloxan RR.

The glucuronic acid results are not complete enough to eliminate this as the possible alloxan RR. Glucuronic acid is unstable and does not exist in the free state in nature. It is found mainly in the conjugated form in blood and urine. This conjugation apparently occurs as a detoxication mechanism and many compounds such as drugs are excreted in this form (4). No confirmation was provided, however, by either the colorimetric or chromatographic results that glucuronic acid or glucuronides were involved. Both, if present, should have been detectable by the silver nitrate procedure. While this indicates that such compounds probably do not form part of the alloxan RR, further investigation is required into the role they play in the normal RR.

On the basis of the chromatographic results a number of conclusions may be drawn about the RR in normal blood. Most of it appears to be one compound only (X). In none of the solvents, under any of the experimental conditions, did it appear that this spot represented more than one compound although the possibility cannot be excluded. Besides this compound, several other compounds appeared occasionally in small amounts. The high R_f value compound appeared only in alcohol-treated blood samples and not in those treated with tungstic acid and pyridine. It is likely that this material was precipitated by tungstic acid and so did not make up part of the Folin reducing substances. These results will be more satisfactory when the compounds are identified.

The chromatograms of blood after alloxan were very similar to those of normal blood. No new reducing compounds and no significant increase in the amounts of those normally present was found by the methods used. This was the case even when the blood sugar determinations

indicated that large amounts of the RR were present.   The compound X
did not appear to be involved in the alloxan RR.   As in normal blood,
several unidentified reducing compounds were present in small amounts.
That some changes did occur was apparent from the appearance of the
chromatograms.   However, nothing detected on the papers could account
for the large amount of RR known to be present.   This is the most
significant aspect of these results.

The disappearance of the ninhydrin-positive material from the
blood after alloxan injection was possibly due to the Strecker reaction,
the deamination and decarboxylation of amino acids by alloxan.   Accord-
ing to Brückmann (16), only one of the resulting products is reducing.
Many of the samples concentrated for chromatography exhibited the pink
colour which results from the reaction.   Pink spots were found on the
chromatograms in some cases, but were not positive to silver nitrate or
ninhydrin.   If free amino acids in the blood were deaminated and decarbo-
xylated, this may be why they could not be detected in normal amounts
by ninhydrin.   These results suggest another way in which alloxan is
inactivated in the blood.

The methods used for paper chromatography have their limitations.
In this case, they do not appear to have led to any serious error.   A
good number of blood samples prepared by two different methods were
run in several solvents.   The R_f values were quite consistent, although
of not much value at this stage.   Unfortunately they cannot be compared
with values in the literature since the experimental conditions differ.

Although further identification of the compounds found will take considerable time, paper chromatography appears to be the best approach to the problem.   Very few techniques are sensitive enough to deal with the small amounts of material found here.   It is hoped that further work will provide more information.

The results of the blood sugar analysis and paper chromatography may be summarized as follows.   A rapid temporary increase in blood RR after alloxan injection has been clearly demonstrated.   The RR in normal blood appears to consist mainly of one compound and small amounts of a few others.   These remain essentially unaltered after alloxan (although the ninhydrin-positive materials decrease).   The increase in alloxan RR is not detectable on the paper by silver nitrate.   This indicates that the increase is not due to a simple reducing sugar and is likely not due to a simple reducing compound at all.

There are several possible reasons why the alloxan RR material cannot be detected on the paper by silver nitrate.   It may be insoluble at the concentration used for spotting, although this seems unlikely since the volume was decreased only to one-tenth of the original.   The following two explanations seem more reasonable.   While normally reducing, the RR material may no longer be reducing on the paper because of changes in the conditions such as dryness or pH.   Alternatively, the material may not normally be reducing but have been altered during the Folin procedure to a reducing form.   With these suggestions in mind, speculation about the nature of the alloxan RR may be profitable.

Three possible explanations may be made for the nature of the
alloxan RR.   The material may be the result of the combination of
alloxan with other compounds in the blood.   As mentioned already, alloxan
reacts with glutathione and amino acids.   Probably it reacts with
other compounds as well and one or more of these may be reducing.   Allo-
xan and dialuric acid (an alloxan derivative) are both reducing although
the former is very unstable.   Although it has been suggested that alloxan
is not likely to be involved since the response did not appear the same
in female toadfish (61), this possibility cannot be excluded.

The second possibility is that the increase in the alloxan RR
may be due to some other non-carbohydrate reducing material.   This
cannot be ignored although no suggestion as to how its presence may be
determined can be offered.

The third possibility is that the increase in the alloxan RR
is carbohydrate.   There is some evidence for this view.   As noted
from the graphs, the glucose level rose only when the RR level started
to drop.   The two may be related.   It is obvious too that alloxan
does produce a disruption of carbohydrate metabolism in some way.
The alloxan RR could result from this.   Furthermore, it can be
pointed out that Falkmer believed that the large amount of RR he found
under various experimental conditions was carbohydrate.   However,
Falkmer's RR and that found here may not be the same.   The possibility
that the alloxan RR is carbohydrate is an interesting and thought-
provoking one.

If the alloxan RR is carbohydrate, then its appearance during the Folin procedure is not surprising. During this procedure by which the RR is measured, the samples, in an acid medium, are boiled fifteen minutes. Under these conditions, hydrolysis could occur resulting in reducing units being produced from non-reducing compounds. Whether this is what actually occurs is not known, but it is a possible explanation. If this is the case the alloxan RR may prove to be quite significant in carbohydrate metabolism.

The alloxan RR may also be important in alloxan diabetes. It may be involved in the initial alloxan reaction on the pancreas which results in beta cell damage and hyperglycemia. This reaction is not understood. It has been suggested that alloxan inhibits vital enzyme systems and that the beta cells are damaged where other cells are not because they contain few sulphydryl compounds which protect against alloxan (45). Little direct evidence has been found for this hypothesis. Lazarow and his group have recently proposed that alloxan affects the permeability of the beta cell membrane and does not actually enter the cell (20, 78). There is insufficient evidence to show how the alloxan RR can be explained by either hypothesis, but any explanation of alloxan action must be able to account for it. It is also possible that the alloxan RR may be involved in some of the reactions attributed to alloxan. Certainly the fact that it is produced indicates again the general toxic nature of alloxan.

Besides providing more information about alloxan action, the results have more practical benefits. They suggest that previous alloxan studies, where reducing methods have been used, require reexamination. The usual blood sugar curve after alloxan is triphasic, an initial hyperglycemia, a drop to hypoglycemic levels, and then permanent or prolonged hyperglycemia (50). Some of the initial hyperglycemia in fish is undoubtedly RR rather than glucose. This may be the case in other animal groups as well. Certainly all future experimental and clinical blood glucose determinations should be conducted using glucose oxidase procedures which are simpler and more accurate. This investigation has indicated how much in error reducing procedures can be.

Several further investigations are required. The identity of X since it is the major component of the normal RR should be determined. This may be accomplished by more chromatography which may also result in the determination of the alloxan RR using different kinds of indicators. Hydrolysis of blood from alloxan treated fish may possibly indicate whether reducing compounds can be produced this way. Investigations should also be made of the behaviour of the RR after alloxan in the blood of animals of different groups. Such studies may prove very valuable. There are many aspects of the problem which require examination and are very interesting.

This investigation has revealed something of the complexity of the alloxan reaction and confirmed the view that the beta cells are not the only target of alloxan. Although the RR, before and after alloxan, has not been identified, considerable information has been provided about

it.   In view of its probable significance in carbohydrate metabolism and alloxan diabetes, it is hoped that the questions raised by this investigation will soon be answered by further research.

Summary

1.   The non-glucose reducing substances were determined in the blood
of toadfish and catfish by measuring the difference between the Folin
value for reducing substances and the glucose value (glucose oxidase).

2.   The rest-reduction in normal blood was 17 mgm % in toadfish and
14 mgm % in catfish.   The glucose values were 19 and 22 mgm % respec-
tively.

3.   After alloxan injection, the RR level increased rapidly to 103
mgm % at one hour in toadfish and 80 mgm % in catfish.   At the same
time, the glucose level had increased only to 23 and 36 mgm % respec-
tively.

4.   At three hours after injection, the RR level had decreased and the
normal level was reached before 24 hours.   The glucose level appeared
to increase to a true hyperglycemia.

5.   The normal blood RR was shown by chromatography to be mainly one
unidentified compound.   A few other reducing compounds were present in
small amounts.

6.   The chromatograms of blood after alloxan were similar to those of
normal blood, although changes were apparent.   The ninhydrin-positive
compounds decreased in number and quantity or the chromatograms,
possibly due to the Strecker reaction.

7.   The alloxan RR was not detectable on the chromatograms by silver
nitrate.   Thus it appeared that simple reducing carbohydrates were not
involved.

8.   It was suggested that the alloxan RR may be a bound carbohydrate of some form.

9.   Future approaches to this problem were suggested as well as other problems to be investigated.

Appendix

Blood Sugar Determinations

Folin-Murrell

This procedure is basically that of Folin and Malmros [28] with some modifications and has been thoroughly discussed by Murrell [55, 56] His procedure was followed exactly. Briefly, the method consists of the following. Proteins are precipitated from the blood by tungstic acid. The sugar and other reducing substances reduce alkaline potassium ferricyanide. The ferrocyanide produced is converted to Prussian blue and measured in a colorimeter.

Glucose Oxidase

This procedure depends on the action of glucose oxidase or glucose to produce gluconic acid and hydrogen peroxidide. The peroxidide reacts with a chromogenic hydrogen donor in the presence of peroxidase to produce a coloured compound which is measured in a colorimeter [81]. The enzyme and dye material are present in a preparation made by Worthington Biochemical Corporation, sold under the name "Glucostat". The Glucostat used here was obtained in 1959. Since then the compound has been modified slightly. The procedure of Saifer and Gerstenfeld [69] with a few minor changes was followed.

1. 0.1 ml blood or standard solution was blown through 7.0 ml 0.25% $CdSO_4$.

2. 1.0 ml NaOH (0.12N) was added.

3.   CdOH precipitated out on standing 15 minutes carrying proteins as
well.   This was centrifuged and the supernatant decanted.

4.   2.0 ml Glucostat reagent (prepared according to the manufacturer's
instructions) was added.

5.   This solution was incubated at $37^{o}C$ for 30 minutes (for toadfish
blood) or one hour at room temperature (for catfish blood).

6.   1.0 ml H_2SO_4 (0.5N) was added and shaken.

7.   This was read after 5 minutes in a colorimeter at 420 mμ.

8.   Unknown values were calculated from a calibration curve prepared
from known standard glucose solutions run at the same time.

Paper Chromatography

The technique of paper chromatography for the isolation and
identification of substances was developed from earlier procedures by
Consden, Gordon, and Martin [18] at Cambridge in 1944 using amino acids.
Few major improvements in their technique have been made since then.
Paper chromatography was first applied to mixtures of sugars by Part-
ridge [63] in 1948.   The major works consulted for this investigation
were Block, Durrum and Zweig [13] and Smith [75].   For a detailed descri-
ption of chromatography and the principles behind it, these may be con-
sulted.

The aspects of chromatography which apply directly to this
investigation must be considered since it is difficult for such results
to be duplicated unless the exact conditions under which these results
were obtained are described.   According to Smith [75], any description
of this type of work should include the following:

1. dimensions of the apparatus

2. grade of paper

3. whether the solvent flow is ascending or descending and the length
   of time required

4. volume and composition of the solvent

5. presence of any other liquid or vapour

6. length of equilibrium time if any

7. temperature

8. nature of the mixture and previous mode of treatment.

To these may be added, the choice of indicators used, application of
the spot, and method of interpretation of the results.

Apparatus

The type of apparatus used was decided by the results desired.
To test 12 different solvents at one time, one pint milk bottles were
used.   The solvent was placed in the bottom and a paper 20 x 4 cm was
arranged so that the bottom of the paper was immersed 1/4" in the sol-
vent and top was held by the cork.   This system, although crude was
effective.   Also during the trial stages when many papers were required,
tanks 15" x 11" x 10" made by Shandon Limited, England were used.
Twelve papers 20 x 20 cm at a time were held in a rack which sat in a
tray containing the solvent.   This was very useful for 2-way runs.
For larger sheets of paper, Shandon glass tanks 13 1/2" x 7" x 14 1/2"
were used.   The papers, 31 x 31 cm, were suspended from wires by stain-
less steel clips or time tape with the bottoms immersed 1/4" in the sol-
vent.   With 2 tanks, 10 papers could be run at one time.   This apparatus,
used most frequently, was good for 2-way runs as well as repeated runs

(2X).   For larger pieces of paper, a glass tank, 18" high and 12" in
diameter was used.   Papers, 41 x 28.5 cm, were fastened together at the
edges with time tape to form cylinders and stood upright with the solvent
in the bottom.   It proved useful to have these different types of equip-
ment available.

Paper

All paper used was prepared for chromatography.   Whatman #4
was tried but discarded, and Whatman #1 and #20 only were used.   Whatman
#1 is fast and provides good separation.   It is the most commonly used
paper for all kinds of chromatography.   Whatman #20 is slower and
provides smaller, more discrete spots, but seems to require more material.
The papers were always run in the same direction, as the "machine direc-
tion" affects the flow rate of the solvent.

Preparation of the Sample

The manner of preparing the sample for chromatography is very
important especially if biological material is involved.   It is necessary
to remove other foreign material such as proteins and ions which may
interfere and to have the right concentration of material in the sample
applied.   In this case, the tungstic acid supernatant from the Folin
determination would have been the best to use since it contained the
reducing substances measured.   However, this solution was too dilute
(0.1 ml blood in 10 ml tungstic acid) and tungstic acid, itself reduced
some indicators.   It was decided then to treat the blood some other
way.

One method used took advantage of the fact that sugars are soluble in pyridine and ions are not. This procedure was modified from that of Malpress and Morrison /51/.

1. 0.5 ml blood was bubbled through 4.5 ml tungstic acid (1 ml 0.67N H_2SO_4, 1 ml 10% sodium tungstate, 7 ml distilled water).

2. This was centrifuged and decanted.

3. The supernatant was dried under vacuum.

4. 1 ml pyridine was added.

5. The tubes containing the pyridine solution were held in boiling water for one minute.

6. This was filtered and the filtrate was again dried.

7. The tubes were sealed and stored in the freezer.

8. For spotting, 0.1 ml i-propanol was added.

This method was complicated and there was a strong possibility that some material may have been lost and others introduced. Consequently it was decided to modify some of the earliest trial experiments using only alcohol to precipitate the proteins. The procedure consisted of these steps.

1. 0.5 ml 95% ethanol was added to 0.5 ml blood.

2. This was shaken, centrifuged, and decanted.

3. Again an equal volume of 95% ethanol was added.

4. This was shaken, centrifuged, and decanted.

5. The resulting solution was dried under vacuum.

6. The tubes were sealed and stored under refrigeration.

7. For spotting, 0.05 ml of glass distilled water was added.

This procedure was simple and not likely to introduce errors. However, not all of the interfering materials were removed. Modifications to improve the precipitation would improve it. A similar method using alcohol and ether was employed by Lohman [49]. Since each method was used on one group of fish, a useful basis for comparison was provided.

Standard solutions were prepared of glucose, glucuronic acid, oxidized and reduced glutathione, uric acid, and alloxan. These were run separately and together. The solutions were prepared to give a concentration similar to that of glucose and the RR in blood. The standard solution was run either on the same paper or at the same time for comparison.

Application of Sample

Hand drawn capillary tubing was used. The spots were about 5 mm in diameter. The material was applied 1" from the bottom of the paper. Since the sample was still very dilute repeated spots were applied. A stream of warm air from an electric hair dryer was used to speed drying. The volume applied was calculated approximately by comparison with the number of spots of similar size from a calibrated pipette. Since this work was qualitative rather than quantitative a high degree of accuracy seemed unnecessary.

Solvent System

This is one of the most critical phases. The degree of separation achieved depends on it to a very large extent. The use of several solvents frequently gives better results.

The following solvents were tried: (V/V)

1. butanol:water 15:1 (13), 1246:84 (13)

2. butanol:acetic acid:water 4:1:5 (17), 50:25:25 (39)

3. ethyl acetate:acetic acid:water 3:1:3 (13)

4. i-propanol:pyridine:acetic acid:water 8:8:1:4 (36)

5. butanol:pyridine:water 3:2:1 (13), 1:1:1 (54), 9:5:8 (13)

6. n-propanol:water 3:1 (13), 7:3 (13)

7. n-propanol:ethyl acetate:water 6:1:3 (13), 7:1:2 (13)

8. collidine:lutidine:water 1:1:1 (13), 1:1:2 (13)

9. butanol;ethanol:water:ammonium hydroxide 40:10:49:1 (13)

10. butanol:ethanol:water 10:1:2 (13)

11. benzene:butanol:pyridine:water 1:5:3:3 (13), 4.5:50:30:30 (15)

12. amyl alcohol:pyridine:water 1:1:2 (13)

13. phenol:water 100:39 (13), 4:1 (13), 264:24 (13)

14. ethyl acetate:pyridine:water 2:1:2 (13)

15. ethanol:butanol:water:cyclohexylamine 10:10:5:2

16. methanol:water:pyridine 80:20:4 (75)

17. n-butanol:formic acid:water 15:3:2 (13)

18. n-propanol:benyl alcohol:water:85% formic acid 50:72:20:20 (32)

19. butanol:ethanol:water:acetone 5:4:2:3 (23)

Of these the most useful are:

4. i-propanol, pyridine, acetic acid:water, PPAW;

5. butanol, pyridine, water (1:1:1) BPW;

11. benzene, butanol, pyridine, water (4.5:50:30:30) BBPW;

7. n-propanol, ethyl acetate, water (6:1:3) PEAW;

19. butanol, ethanol, water, acetone BEWA.

PPAW gives the best separation of any solvent tried and is reasonably fast.

Two main procedures were used. Most frequently the papers were run in one solvent, dried, and then run in another so that the second solvent flowed at right angles to the first. This is referred to as 2-way chromatography. Later, it was useful to run the chromatograms the second time in the same direction in the same solvent. This is referred to as repeated or 2X chromatography. It has been recommended by Jermyn and Isherwood (42) and Pasieka (64) to eliminate the salt effect and provide better separation (see Plates 4-8).

The temperature at which the papers are run is important for reproducibility. It was always recorded, but could not be controlled and fluctuations occurred. Equilibrium of the paper in the solvent vapour was not carried out. The solvent, always the same amount, was allowed to vapourize in the container for at least 24 hours before use and was made up fresh frequently. The solvent was allowed to flow to about 1/2" from the top, but variations occurred. When complete, the papers were hung up to dry at room temperature for several hours before the next step was carried out.

Indicators

Almost all of the indicators tried were not sensitive enough for the small amounts involved. The following were tried:

1. ammonia silver nitrate (13) (75)

2. potassium permanganate (36)

3. ammonium molybdate (13)

4. dinitrosalicyclic reagent (75)

5. Benedict's solution (58)

6. Somogyi's reagent (13)

7. Folin reagent

8. periodic-acid benzidine (75)

9. naphthoresorcinol (13)

10. aniline oxalate (13)

11. benzidine-acetic acid (13) (19)

12. benzidine-trichloracetic acid (75)

13. aniline diphenylamine (75)

14. FFCA for iodine containing compounds (33)

15. aniline phthalate (13)

16. glucostat (70)

17. p-anisidine (13)

The only procedure found sensitive enough was alkaline silver nitrate. It is not specific for sugars which was useful here. The procedure followed was basically that of Trevelyan (77) with the modification of Evans and Dethier (23).

1. A silver nitrate solution was prepared by adding 0.5 ml saturated aqueous silver nitrate solution to 100 ml acetone. Water was added a few drops at a time until the precipitate disappeared. This was prepared fresh.

2. The papers were dipped in this and hung to dry.

3. They were next dipped in 0.5N NaOH (90% ethanol).

4.   When nearly dry they were placed in Kodak Acid Fixer for about one minute.

5.   They were washed in running tap water thoroughly, blotted, and hung to dry.

Glucose was located exactly by the method of Salton (70) using the Glucostat reagent.

For detection of amino acids and their derivatives, the papers were dipped in 0.25% ninhydrin in acetone (13).   They were heated at 50°F for one half hour.

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