creased activities of the detoxifying enzymes aniline hydroxylase and nitroreductase. The amount of enzyme activity per cell is reduced, and because of the partial atrophy of the liver following diversion of the portal flow, the total enzyme activity of the liver is even more drastically decreased. In normal animals treatment with phenobarbital results in hypertrophy of the smooth endoplasmic reticulum (6) and increased activities of hepatic drug metabolizing enzymes (7). After treatment with phenobarbital, the liver deprived of portal blood can still respond with a hypertrophy of smooth endoplasmic reticulum and an increase in enzyme synthesis. Although the relative increase in the levels of drug metabolizing enzymes is comparable in both experimental and control animals, the total increase is greater in controls, since their initial levels are higher. No quantitative correlation between the degree of hypertrophy of smooth endoplasmic reticulum and the increased levels of enzymes can be made. The cause for the decreased levels of detoxifying enzymes is not clear. This defect may be a manifestation of liver cell injury, possibly anoxic. An oxygen deficit may interfere with microsomal electron transport mechanisms. Another possibility is that these enzymes are normally induced by products of bacterial flora or of digestion, which are brought to the liver from the intestine via the portal vein. Diversion of the portal flow decreases the concentration of these products and thus may reduce the concentration of an endogenous inducer. The liver does not lose its potential for reacting to an inducer, as manifested by its response to phenobarbital.

It has been suggested that portasystemic encephalopathy after portacaval shunts in man may be caused by a toxic factor which is not detoxified by the liver because of the diversion of the portal flow (3). The data here presented indicate that under these conditions a defect in the detoxifying ability of the liver itself may be an additional factor.

*Summary.* Diversion of the portal flow from the liver in rats results in a reduction of the activities of hepatic drug metabolizing enzymes. Such animals still respond to phenobarbital with an increase in hepatic enzyme levels and hypertrophy of smooth endoplasmic reticulum in hepatocytes. It is suggested that a defect in the detoxifying ability of the liver itself may play an additional role in portasystemic encephalopathy in man.

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## Inhibition of the Esterase Activity of Thrombin by $Na^+$* (32712)

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Although it is well known that $Na^+$ and $K^+$ have opposing effects on the activities of several enzymes, to our knowledge this has not been reported to be the case with any of the proteins involved in the clotting of the blood. We are now reporting that $Na^+$ inhibits the hydrolysis of TAME(*p*-toluenesulfonyl-L-arginine methyl ester) by both human and bovine thrombin, while $K^+$ does not.

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*Materials and Methods.* Four preparations

TABLE I. Effect of KCl and NaCl on the Hydrolysis of TAME by Preparations of Thrombin. Rates in the Presence of from 0.1 to 0.4 *M* KCl (Controls) Were the Same and Were Set Equal to 100%.

| Thrombin | Salt (molarity) KCl | Salt (molarity) NaCl | Percentage of control rate |
|---|---|---|---|
| Bovine, undialyzed | | | |
| 4 units/ml | 0.10 | 0.10 | 63 |
| | 0.10 | 0.20 | 57 |
| 8 units/ml | 0.10 | 0.10 | 70 |
| | 0.10 | 0.20 | 62 |
| | 0.10 | 0.40 | 63 |
| Bovine, dialyzed | | | |
| 8 units/ml | None | 0.09 | 61 |
| Human, undialyzed | | | |
| Fibrindex, 2 units/ml | 0.10 | 0.10 | 64 |
| | 0.20 | 0.10 | 70 |
| Aronson, 0.04 mg/ml | 0.10 | 0.10 | 65 |
| Miller, 8 units/ml | None | 0.16 | 59 |
| Human, dialyzed | | | |
| Miller, 8 units/ml | None | 0.16 | 48 |

of thrombin were tested: Topical Bovine thrombin (Parke-Davis), Human Fibrindex (Ortho Pharmaceutical Corp.), and 2 highly purified human thrombins, kindly given to us by Dr. David L. Aronson, Division of Biologics Standards, U. S. Public Health Service and by Dr. Kent Miller, Division of Laboratories and Research, N. Y. State Depts. of Health. Each was dissolved in cold 50% glycerol–water (v/v) and either immediately placed in the freezer compartment of a refrigerator or dialyzed for 1 week against frequent changes of cold 50% glycerol–water to remove salts, sugar, and preservatives, and then placed in the freezer. Just before testing, aliquots were removed and diluted with the desired salt.

Trypsin and soybean trypsin inhibitor (SBTI), salt-free and recrystallized preparations, were purchased from Worthington Biochemical Corp. All the other chemicals were the purest grade commercially available.

The rates of hydrolysis of TAME by either trypsin or thrombin were determined by a modified Hestrin method (1,2). All tests were performed in duplicate at 37°. There was never any significant hydrolysis of TAME in the absence of enzyme. At the start of the rate measurements all solutions contained 0.02 *M* TAME and, unless otherwise stated, 0.25 *M* Tris-HCl buffer (pH 7.8).

*Results and Discussion.* Table I shows that the rates of hydrolysis of TAME by all 4 thrombin preparations were inhibited from 30 to 52% (av 38%) due to the presence of from 0.09 to 0.4 *M* NaCl, regardless of whether KCl was absent or present in the tests. From 0.1 to 0.4 *M* KCl alone had no significant effects on the rates. With lower concentrations of NaCl, 0.002 and 0.005 *M* (not shown in the table), the rates were inhibited 13 and 25% respectively, in the presence of both 0.02 and 0.1 *M* KCl. These results were obtained when using either Baker Analyzed Reagent or Fisher Certified Reagent NaCl.

If the inhibition found were caused by impurities present in both lots of NaCl, a different sodium salt might not produce any inhibition. Two phophate buffers of the same pH and molarity were therefore prepared. One was made from the mono- and disodium phosphates, the other from the corresponding potassium salts. The data in Table II show that the rates of hydrolysis of TAME by either dialyzed bovine or human (Miller) thrombin in the presence of 0.2 *M* potassium phosphate buffer (pH 7.40) were double those found in 0.2 *M* sodium phosphate buffer (pH 7.45).

The following experiments were performed to determine whether $Na^+$ inhibited throm-

TABLE II. Rates of Hydrolysis of TAME by Thrombins in the Presence of a Potassium or a Sodium Buffer of the Same pH and Molarity.

| Buffer (0.2 *M*) | TAME hydrolyzed in 30 min/ml test (μmoles) Dialyzed thrombin: Human (Miller) | Bovine |
|---|---|---|
| Potassium phosphate, pH 7.40 | 17.4 | 16.4 |
| Sodium phosphate, pH 7.45 | 8.72 | 8.40 |

TABLE III. The Effect of Varying the Concentration of Glycerol on the Hydrolysis of TAME by Dialyzed Bovine Thrombin in the Presence and Absence of NaCl.

| Glycerol (%) | TAME hydrolyzed in 30 min/ml test (μmoles) 0.2 *M* KCl | 0.1 *M* KCl plus 0.1 *M* NaCl | (Rate in NaCl × 100)/(Rate in KCl) (%) |
|---|---|---|---|
| 1 | 17.3 | 10.2 | 59 |
| 11 | 11.2 | 5.12 | 46 |
| 21 | 8.10 | 4.10 | 51 |
| 31 | 5.34 | 2.56 | 48 |
| 41 | 4.60 | 2.76 | 60 |

bin or another enzyme, contaminating all 4 thrombin preparations:

1. Thrombin is inhibited by glycerol but a contaminating enzyme might not be. Table III shows that increasing the concentration of glycerol from 1 to 41% decreased the rates of hydrolysis of TAME by dialyzed bovine thrombin, both in the presence and absence of 0.1 *M* NaCl. At each glycerol concentration the rates in NaCl averaged 53% of those found in its absence. A contaminating enzyme, if present, was inhibited to the same degree as was thrombin by varying concentrations of glycerol.

2. Thrombin is unstable in acid solution but a contaminating enzyme might not be. Table IV shows that dialyzed bovine thrombin, incubated at 37° and pH 3.4 rapidly lost activity. At each time of incubation, the rates of hydrolysis of TAME in the presence of NaCl averaged 54% of the rates found in the presence of KCl. The postulated enzyme, therefore, lost the ability to hydrolyze TAME at the same rate as thrombin did.

3. Thrombin is not inhibited by SBTI, but a contaminating enzyme might be. It was first established that rates of hydrolysis of TAME by trypsin were the same in the presence of 0.2 *M* KCl or NaCl, and that SBTI totally inhibited the hydrolysis in both cases. These experiments served as a check on all the solutions and the testing method and also eliminated trypsin as a possible contaminant in the thrombin preparations.

The hydrolysis of TAME by dialyzed bovine or human (Miller) thrombin was not inhibited by SBTI whether the experiments were conducted in 0.2 *M* KCl or NaCl. With NaCl present the rates were 58% of those found with KCl present, both in the presence and absence of SBTI. All tests were performed using 0.05 mg/ml of SBTI (or none), 8 units/ml of thrombin, or 0.0025 or 0.005 mg/ml of trypsin and either 0.2 *M* KCl or NaCl. The postulated contaminating enzyme, therefore, like thrombin, was not inhibited by SBTI.

Previously we had shown that both NaCl and KCl produce the same acceleration of the rates of hydrolysis of TAME by human plasmin (3). This enzyme, therefore, can be eliminated as the contaminant inhibited by $Na^+$.

The data presented strongly indicate that $Na^+$ inhibits the hydrolysis of TAME by thrombin but $K^+$ does not. Whether thrombin's action on fibrinogen is also inhibited by $Na^+$ remains to be determined. The finding, however, of a differential effect of $Na^+$ and $K^+$ on a key enzyme involved in the clotting of blood, makes it important to investigate the effects of these ions on all steps in blood clotting, particularly the early ones.

*Summary.* The hydrolysis of TAME by bovine and human thrombin was inhibited

TABLE IV. The Effect of Incubating Dialyzed Bovine Thrombin at 37° in HCl, pH 3.4, for Varying Times on Its Ability to Hydrolyze TAME when Tested in the Presence of KCl or NaCl. Stock Thrombin Solutions Were Diluted with HCl to pH 3.4 and at Times Shown Aliquots Were Tested for TAME-Hydrolyzing Activity.

| Time at 37° and pH 3.4 (min) | TAME hydrolyzed in 30 min/ml test (μmoles) 0.1 *M* KCl | 0.1 *M* NaCl | (Rate in NaCl × 100)/(Rate in KCl) (%) |
|---|---|---|---|
| 0 | 14.1 | 8.20 | 58 |
| 15 | 11.9 | 6.47 | 54 |
| 30 | 9.95 | 4.86 | 49 |
| 45 | 8.90 | 4.77 | 54 |
| 60 | 7.90 | 4.36 | 55 |

about 40% by 0.1 *M* NaCl but not by even higher concentrations of KCl. Probably $Na^+$, and not an impurity in the NaCl used, produced the inhibition because NaCl from a second source gave the same results, and in addition, rates of hydrolysis when using a buffer made from the mono- and dipotassium phosphates were double those found when using a buffer made from the corresponding sodium phosphates. No evidence was found that $Na^+$ inhibited, not thrombin, but a contaminant in the 4 thrombin preparations tested. Increasing concentrations of glycerol in the tests produced increasing inhibition of hydrolysis, but the percentage inhibition due to $Na^+$ remained constant. Incubating thrombin at 37° and pH 3.4 gradually destroyed its ability to hydrolyze TAME, but at all times tested the percentage inhibition due to $Na^+$ remained constant. SBTI did not inhibit the rates, and the percentage inhibition by $Na^+$ was the same in the presence and in the absence of SBTI. For these reasons it was concluded that thrombin itself is inhibited by $Na^+$.

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## Growth Hormone, Plasma Glucose, and Ketone Bodies as Determinants of Cardiac Glycogen in Normal and Diabetic Rats* (32713)

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An impressive body of evidence has accumulated indicating that both cardiac metabolism and cardiac contractile activity rely preferentially on noncarbohydrate sources of energy. That fatty acid metabolism is most closely linked with such energy demands has been indicated in many studies carried out on mammalian hearts both at rest and during exercise, employing *in vitro* and *in vivo* techniques (1–6).

Elevated cardiac glycogen levels in rats resulting from feeding free fatty acids (7,8), during fasting (9), and in diabetes (8,10) have been observed; the need for adequate circulating growth hormone for such a polysaccharide increase during fasting has been well defined (9,11). Not all studies, however, have divorced plasma ketone levels from plasma glucose levels as possible influencing factors in regulating cardiac glycogen concentrations. Furthermore, the observation that the diabetic rat hypophysis contains one sixth the normal amount of growth hormone (12, 13) is yet to be reconciled with the elevated heart glycogen levels frequently reported in such animals (10).

It appeared of interest to undertake studies which would attempt to clarify the interrelationships among cardiac glycogen, plasma glucose, and plasma ketone levels. Also attempts were made to assess cardiac polysaccharide responses to exogenous growth hormone in the fasted and nonfasted diabetic rat.

*Methods.* All rats used were male Cheek-Jones (Houston) rats derived from the Holtzman strain, kept at 75±1°C and fed Purina laboratory chow and water *ad libitum*. Blood and urinary glucose were determined by the Nelson-Somogyi colorimetric method (14), tissue glycogen by the anthrone method (15), and total and differential plasma ketones (details by personal communication) by the method of Bessman and Anderson (16). In this ketone method double oxidation of acetoacetate and β-hydroxybutyric acid by sulfuric acid and dichromate allow for estimation of β-hydroxybutyric acid by subtracting acetoacetate levels from those of total acetone. Plasma protein was determined by Lowry

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