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The Effects of Salicylate and Adrenocortical Preparations Added *in vitro* upon the Glycogen Content of Liver Slices

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The injection of salicylate has been observed to cause a marked depletion of the liver-glycogen level in normal rats (Lutwak-Mann, 1942; Smith, Meade & Bornstein, 1952) and to prevent the deposition of liver glycogen induced by the injection of cortisone in adrenalectomized rats (Smith, 1952). The present paper is concerned with the effects of salicylate and adrenocortical preparations added *in vitro* on the glycogen content of rat-liver slices incubated in buffered physiological salt solutions.

EXPERIMENTAL

Preparation of tissue. Male Wistar rats, weighing 150–300 g., maintained on a diet of cubes (Medical Research Council diet 41) were used. The rats were killed by stunning, the liver was removed and slices of thickness 0.3–0.4 mm. were prepared with a scalpel, always from the same lobe. The slices as cut were placed serially in a number of piles, equal to the number of samples needed, in a Petri dish over ice. Each sample, weighing 80–100 mg., consisted of about four slices. The samples were weighed at the beginning of the experiment on a torsion balance. Samples in duplicate were used throughout and the average difference between duplicates for the glycogen estimations was $\pm 6.3\%$.

Conditions of incubation. Two salt solutions were used: (a) phosphate-Ringer (Seckel, 1940): 8.0 g. NaCl, 1.0 g. NaHCO₃, 0.1 g. KCl and 0.1 g. CaCl₂, H₂O were dissolved in 1 l. of distilled water; 90 ml. of this solution were mixed with 7.0 ml. $m/15$ Na₂HPO₄ solution and 3.0 ml. $m/15$ NaH₂PO₄ solution before use; (b) solution containing K 110, Mg 20, Ca 10, HCO₃ 40 and Cl 100 m-atoms/l. (Hastings, Teng, Nesbitt & Sinex, 1952). The initial pH was 7.4 in all cases, an atmosphere of air or O₂ being used with (a) and 95% O₂–5% CO₂ (by vol.) with (b).

The samples, each with 2 ml. of medium, were placed in Warburg flasks which were attached to manometers and shaken continuously at 37°. At the end of the experiment the slices were transferred as rapidly as possible to 30% (w/v) KOH. The slices for initial values of glycogen were placed in 30% (w/v) KOH at the time the experimental samples went into the bath.

Materials. Salicylic acid (A.R.) was dissolved in the salt solutions to give final concentrations between 0.005M and 0.02M. 0.005M salicylate (69 mg./100 ml.) was chosen as a suitable initial concentration because plasma salicylate concentrations of 50–70 mg./100 ml. were found in rats at the time when maximum liver-glycogen depletion occurred following the subcutaneous injection of sodium salicylate.

The adrenocortical extract used was Eschatin, free from preservative (Parke, Davis and Co.), 1 ml. of which was

equivalent to 90 g. fresh adrenals. The amount used was 0.3 ml. for each flask (0.15 ml./ml. medium), as this quantity was reported by Chiu & Needham (1950) to give a maximum effect in decreasing glycogen disappearance.

Cortisone and hydrocortisone alcohols were dissolved in Seckel's phosphate-Ringer solution to give final concentrations ranging from 30 to 60 μ g./ml. medium in the Warburg flasks. During the early part of the work, the commercial suspension of cortisone acetate (Cortone acetate, Merck) was used, but a clear solution could not be obtained at dilutions of less than 1 in 200 (12.5 μ g. cortisone acetate/ml.). No effect on glycogen breakdown in the liver slices was obtained with this concentration and the free cortisone alcohol, which is 14 times as soluble as the acetate (Macek, Baade, Bornn & Bacher, 1952) was used to produce the higher concentrations.

Analytical methods. Glycogen was separated by the method of Good, Kramer & Somogyi (1933), the glucose in the final stage of the estimation being measured by the method of Nelson (1944). The Eschatin was analysed for cortisone and hydrocortisone by the method of de Courcy, Bush, Gray & Lunnion (1953) and for potassium and sodium by the flame photometer.

RESULTS

The results in Table 1 show the effect of the addition of salicylate on the glycogen disappearance from liver slices incubated in Seckel's phosphate-Ringer

solution in an atmosphere of air. The results are expressed as percentages of the glycogen level at the beginning of the experiment. This initial glycogen content was 1.34–5.10 g./100 g. wet tissue.

Comparison of the results by Student's *t* test showed that there was no significant difference between the amounts of glycogen broken down in the control experiments and in the flasks containing salicylate concentrations up to 0.02M (276 mg./100 ml.).

When the experiments were repeated substituting the salt solution of Hastings *et al.* (1952) for Seckel's phosphate-Ringer and incubating for 60 min. under an atmosphere of 95% O₂–5% CO₂, it was found that the addition of 0.005M salicylate caused a significant increase in glycogen disappearance ($t = 2.8909$, $P = 0.02$) in the slices. The results from eight rats, expressed as percentages of the initial glycogen content of the slices, were 48.2 ± 7.9 (range, 34.3–55.8) for the control and 37.4 ± 7.0 (range, 23.7–45.0) for the flasks containing 0.005M salicylate.

As shown in Table 2, Eschatin caused a significant decrease in glycogen disappearance in the absence, but not in the presence, of 0.005M salicylate.

Table 1. *The effect of salicylate on glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were suspended in 2 ml. of Seckel's phosphate-Ringer under an atmosphere of air and incubated for 30 or 60 min. at 37° with shaking. Mean values $\pm$ standard error and ranges are given for the results, which are expressed as percentages of the initial glycogen content.

Molar concentration of salicylate	No. of rats	30 min.		60 min.	
		Control	Salicylate	Control	Salicylate
0.005	6	48.1 ± 20.0	46.8 ± 20.1	31.6 ± 18.0	31.3 ± 18.4
	Range	22.2–70.5	20.8–65.8	9.0–51.0	9.0–51.4
0.01	7	58.2 ± 19.2	51.2 ± 19.7	39.4 ± 19.9	32.3 ± 16.9
	Range	36.6–84.1	29.2–78.7	13.4–66.8	10.4–58.8
0.02	9	50.0 ± 15.1	42.7 ± 15.4	28.2 ± 14.1	21.5 ± 12.3
	Range	30.2–67.7	27.0–66.4	15.5–55.5	11.9–47.3
<i>Statistical significance of the differences between the results for the control and salicylate groups</i>					
0.005M	—	$t = 0.1119$	$P = 0.9$	$t = 0.0285$	$P = 0.9$
0.01M	—	$t = 0.6715$	$P = 0.6$	$t = 0.7169$	$P = 0.5$
0.02M	—	$t = 1.0123$	$P = 0.4$	$t = 1.0713$	$P = 0.3$

Table 2. *The effect of Eschatin, with and without 0.005M salicylate, on the glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were suspended in 2 ml. of Seckel's phosphate-Ringer under an atmosphere of oxygen and incubated for 60 min. at 37° with shaking.

No. of rats	% of initial glycogen content after incubation			
	Control (A)	Eschatin (B)	Salicylate (C)	Eschatin + salicylate (D)
6	19.2 ± 9.5	32.3 ± 7.3	18.6 ± 7.0	21.4 ± 6.8
Range	12.5–29.2	22.1–45.4	8.7–28.9	12.2–34.3

Statistical significance of differences between results

For groups A and B: $t = 2.6974$, $P = 0.02$; for groups C and D: $t = 0.6541$, $P = 0.5$.

Table 3. *The effect of Eschatin, cortisone and hydrocortisone on glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were incubated in 2 ml. Seckel's phosphate-Ringer under an atmosphere of oxygen for 60 min. at 37° with shaking. Cortisone and hydrocortisone present in concentrations of either 30 or 60 µg./ml. medium.

No. of rats	% of initial glycogen content after incubation					
	Control	Eschatin	Cortisone		Hydrocortisone	
			(30 µg./ml.)	(60 µg./ml.)	(30 µg./ml.)	(60 µg./ml.)
7	31.0 ± 7.9	49.6 ± 11.2	29.8 ± 8.3	29.4 ± 7.7	28.5 ± 7.0	26.7 ± 6.2
Range	18-40	29-61	16-42	17-38	16-35	16-34

Statistical significance of differences between results

For control and Eschatin: $t = 3.5876$, $P = 0.01$; for control and cortisone, and for control and hydrocortisone: $P > 0.3$ in all cases.

As shown in Table 3, although Eschatin caused a significant decrease in glycogen disappearance, cortisone and hydrocortisone in concentrations of 30 and 60 µg./ml. medium had no effect. Analysis of the Eschatin for adrenocortical steroids by the method of de Courcy *et al.* (1953) showed that the extract contained approx. 100 µg./ml. of cortisone and 50 µg./ml. of hydrocortisone. 0.15 ml. Eschatin/ml. medium was therefore equivalent to approx. 16 and 8 µg. respectively of the two steroids/ml. of medium in the Warburg flasks. The Eschatin contained 0.8 mg./100 ml. of K and 370 mg./100 ml. of Na.

DISCUSSION

When liver slices are incubated in buffered physiological salt solutions in air or oxygen the observed degree of glycogen disappearance is the resultant of simultaneous breakdown and synthesis. The importance of the cationic composition of the incubation medium has been emphasized by Hastings *et al.* (1952), who demonstrated that the sodium/potassium ratio in particular affected the amount of glycogen formation by rat-liver slices. A high sodium/potassium ratio decreased the amount of new glycogen formed whereas a low sodium/potassium ratio produced the reverse effect.

The two buffered salt solutions used in the present work differed in that Seckel's phosphate-Ringer contained mainly sodium salts with a very small proportion of potassium salts, whereas the solution of Hastings *et al.* contained no sodium and a high proportion of potassium. When the liver slices were incubated in these two solutions, more glycogen disappeared in the former than in the latter, the average results after 60 min. incubation, expressed as percentages of the initial glycogen content, being 33.0 and 48.2 respectively. If the difference was due to greater glycogen formation occurring in the slices incubated in the solution of Hastings *et al.*, then the observed effect of added salicylate in causing more glycogen disappearance in this

solution, but not in Seckel's phosphate-Ringer, may be caused by an interference with the processes concerned with the synthesis rather than the breakdown of glycogen. This *in vitro* effect was produced at salicylate concentrations similar to those observed in the blood of normal rats showing marked depletion of liver glycogen after injection of salicylate. Interference with glycogen synthesis in the liver may therefore contribute to this depletion *in vivo*.

Seckel (1940) reported that addition of adrenocortical extract (Eschatin, free from preservative) to phosphate-Ringer solution in which liver slices were incubated caused a decrease in glycogen disappearance. His observation was confirmed by Chiu & Needham (1950), who showed that the Eschatin increased the formation, rather than decreased the breakdown, of glycogen. The present results again confirm the work of Seckel and also show that the addition of 0.005M salicylate inhibits the increased glycogenesis induced by the adrenocortical extract. The possibility was considered that Eschatin might contain a high proportion of potassium salts and that the addition of 0.3 ml. to 2 ml. of Seckel's phosphate-Ringer might increase the potassium/sodium ratio in the final incubation medium, thus causing increased glycogenesis. However, the extract was found to contain only a small concentration of potassium and a relatively large amount of sodium and this explanation was therefore not valid.

The next series of experiments was designed to determine if the antagonism between salicylate and the adrenal-cortex extract could be extended to pure adrenocortical steroids, because Chiu (1950) claimed that certain steroids, including cortisone, acted similarly to Eschatin *in vitro*. However, no effect of cortisone or hydrocortisone, in concentrations greater than those employed by Chiu (1950) and present in the Eschatin, on glycogen disappearance in rat-liver slices, could be demonstrated. Verzář (1951) has also reported that he did

not succeed in obtaining significant glycogen production with rat-liver slices incubated with corticosteroids *in vitro* in the presence of glucose or pyruvate as substrates. The present results indicate first, that the well-marked *in vitro* action of Eschatin cannot be attributed to its content of cortisone or hydrocortisone and, secondly, that passage of cortisone through the body is necessary for the injection of the hormone to produce liver-glycogen deposition in adrenalectomized rats. The *in vitro* antagonism of salicylate and Eschatin observed in the present work and the effect of salicylate in preventing the deposition of liver glycogen induced by cortisone in the adrenalectomized rats (Smith, 1952) therefore cannot be explained on a common basis.

SUMMARY

1. The effects of the addition of salicylate, adrenocortical extract, cortisone and hydrocortisone on the glycogen content of rat-liver slices incubated in buffered physiological salt solutions have been investigated.

2. Salicylate caused a significant increase in the glycogen disappearance in liver slices incubated in a medium containing a high concentration of potassium and no sodium, but not in a medium containing a high proportion of sodium.

3. Adrenocortical extract (Eschatin, free from preservative) but not cortisone or hydrocortisone,

produced a significant decrease in the glycogen disappearance and this effect was inhibited by the presence of salicylate.

4. The implications of these effects are discussed.

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The Action of Polylysine on the Conversion of Fibrinogen into Fibrin by Coagulase Thrombin

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It has been shown previously that polylysine, a synthetic basic water-soluble poly- α -amino acid, retards blood coagulation and this was shown to be due to interference with the formation of thrombin from prothrombin (de Vries, Schwager & Katchalski, 1951). The present study was undertaken to investigate a possible effect of polylysine on the clotting of plasma by staphylocoagulase. Polylysine was found to accelerate this clotting reaction.

The coagulation of plasma by staphylocoagulase proceeds in two stages: (a) staphylocoagulase reacts with a coagulase-reacting factor present in plasma to form coagulase thrombin, (b) coagulase thrombin converts fibrinogen into fibrin (Miale, 1949; Tager, 1948). The action of polylysine on the

clotting of fibrinogen in the presence of staphylocoagulase and coagulase-reacting factor was therefore investigated.

EXPERIMENTAL

Materials

Staphylocoagulase. This was obtained by the method of Duthie & Lorenz (1952) from a strain of *Staphylococcus aureus*, Newman (NCTC 8178), grown in broth or in brain-heart infusion for 24 hr. The organisms were removed by centrifuging in a Sorvall centrifuge for 15 min. at 12 000 rev./min. at 4°.

Coagulase-reacting factor. This was obtained from pooled oxalated human plasma according to the method of Kaplan & Spink (1948), and stored in 0.9% NaCl at 4°.