EFFECT OF EPINEPHRINE ON GLUCOSE METABOLISM IN NOVIKOFF ASCITES HEPATOMA CELLS

By

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ABSTRACT

No major effects of epinephrine on glycogen synthesis and glycolysis by intact Novikoff ascites-hepatoma were noted. However, at $2 \times 10^{-5}$ M epinephrine concentration glycogen synthesis from glucose was slightly enhanced and lactic acid production was slightly decreased. These effects were attributed to a stimulation of UDPglucose-$\alpha$-glucan glucosyltransferase and an inhibition of phosphofructokinase. Breakdown of glycogen deposited by the tumour cells was also inhibited to a small extent by epinephrine.

The pattern of epinephrine action on glucose metabolism of tumour cells suggests that loss as well as modification of the receptor (of epinephrine) occurs during neoplastic transformation of liver.

Administration of epinephrine into animals normally results in the mobilisation of liver and muscle glycogen and elevation of blood sugar level. Sutherland and his coworkers have proposed that membrane bound adenylyl cyclase is the primary adrenergic receptor in animal tissues (Robinson et al. 1967). Glycogenolytic action of epinephrine is believed to occur in a stepwise manner. First, activation of adenylyl cyclase results in increased conversion of ATP into adenosine 3′,5′-phosphate (cyclic AMP$^{\ast\ast}$). Cyclic AMP, in turn, augments

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$^{\ast\ast}$ The abbreviations used but not described in full in the text are: AMP and ATP, adenosine 5′ mono- and tri-phosphate; UDPG or UDPglucose, uridine diphosphate glucose.
phosphorylase kinase activity. Activated phosphorylase kinase transforms phosphorylase $b$ (inactive) into phosphorylase $a$ (active) in the presence of ATP and Mg$^{2+}$. In consequence, glycogen is broken down by phosphorylase $a$ into glucose 1-phosphate and activation of phosphofructokinase by cyclic AMP (Passonneau & Lowry 1962) accomplishes transformation of glycogen to lactate along the Embden-Meyerhof pathway. Besides the above sequence for phosphorylase activation increased concentration of phosphorylase $a$ can be obtained by inhibition of a specific phosphodiesterase (Robinson et al. 1967) (which converts cyclic AMP into 5'-AMP) or activation of phosphorylase $b$ by a proteinase in the presence of Ca$^{2+}$ ions (Huston & Krebs 1968). In other studies, it has been observed that activation of phosphorylase by epinephrine is followed by inhibition of UDPglucose-$\alpha$-glucan glucosyltransferase (Belocopitow 1961). Craig & Larner (1964) have further shown that epinephrine action results in the decrease of both glucose 6-phosphate dependent (D) and independent (I) forms of UDPglucose-$\alpha$-glucan glucosyltransferase.

During the course of a study on the glycogen metabolism of Novikoff ascites-hepatoma cells, it became apparent that the unusual ability of this tumour to store glycogen (Nigam 1967a) made it a useful tissue to ascertain the response of epinephrine on its glucose and glycogen metabolism. The present study describes the action of epinephrine on the transformation of glucose and certain glucose-intermediates into glycogen and lactic acid. Absence of glyco-genolytic action, slight activation of UDPglucose-$\alpha$-glucan glucosyltransferase and slight inhibition of phosphorylase demonstrate the emergence of a reversed pattern of hormonal response in this tumour, as compared to rat liver, its tissue of origin.

**MATERIALS AND METHODS**

*Chemicals:* Glucose-1-$^{14}$C was obtained from Merck and Co., Montreal, glucose-1-$^{14}$C 6-phosphate and glucose-U-$^{14}$C 1-phosphate from New England Nuclear Corp., Cambridge, Mass., and UDPglucose-U-$^{14}$C from International Chemical and Nuclear Corp., City of Industry, Calif. Non-radioactive fine chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade purity obtained from commercial sources.

*Animals:* Sprague-Dawley rats weighing 150–200 g were obtained from Canadian Breeding Laboratories, St. Constant, Que. Food and water were supplied to the animals *ad libitum*.

*Preparation of tumour cells:* Rats were transplanted intraperitoneally with 50–70 million Novikoff ascites-hepatoma cells. After 4–7 days, the animals were sacrificed and the ascitic fluid was collected in 4 volumes of ice-cold Tris-wash medium described by Wu & Racker (1959). The tumour cell suspension was centrifuged at low speed (60–80 $\times$ g) for 4–6 min at 0°C in an International refrigerated centrifuge. The sedimented cells were washed 4–5 times with 10 volumes of the cold medium each time to free them from erythrocytes and polymorphonuclear leukocytes (Nigam 1962). The
washed tumour cells were later suspended in Tris-incubation medium of Wu & Racker (1959) and centrifuged at 1200 X g for 8 min in a graduated centrifuge tube to ascertain their volume. The sedimented cells were kept over ice and were diluted with 5 volumes of Tris-incubation-medium prior to their use.

Aging of the tumour cells: Tumour cells were aged by leaving washed tumour cells at 0–5°C for 18–24 h (Nigam 1967b). They were washed once before being used in a metabolic reaction.

Incubation procedure: Tumour cell suspension (0.9 ml) was transferred to 20-ml beakers. Epinephrine solution (0.1 ml) of desired concentration in 10 molar neutral ascorbic acid solution was added. Control reaction mixtures contained 0.1 ml ascorbic acid solution only. After 5 min of incubation at room temperature, 0.05 ml labelled glucose solution (0.1 m, 50 000 counts/min) was added and the beakers were transferred to a Dubnoff metabolic shaker maintained at 36–38°C and flushed with 100% oxygen. The digs were shaken at 10–20 strokes per min. Duplicate digests were usually run and one reaction mixture was used for the isolation of glycogen and the estimation of its 14C and the other for the determination of lactic acid, glucose and sugar phosphates.

Isolation of glycogen: Glycogen was isolated from reaction mixtures by digestion with hot KOH and precipitation with ethanol as described by Robbins et al. (1959), except that 0.1 ml instead of 0.2 ml saturated sodium sulphate was used for the precipitation of glycogen.

Estimation of the radioactivity in glycogen: In most experiments where incorporation of glycogen was to be determined 5 mg unlabelled rabbit liver glycogen was added after KOH digestion. The isolated glycogen was dissolved in 2 ml warm distilled water and 1 ml solution was plated on aluminium planchetts (5 cm² in area) and dried. Radioactivity was measured by using a Picker Nuclear Counter with an ultra thin window. The samples were found to have negligible self absorption in the range of glycogen usually present in the samples (2.5–3.0 mg/ml). It was necessary to correct for absorption due to 0.05 ml saturated sodium sulphate solution present per ml of the sample. It reduced the counts 25%. Since all samples contained the same amount of sodium sulphate, the same correction factor was applied to all samples.

Estimation of the amount of glycogen synthesised: The number of micromoles of glucose equivalent of glycogen formed was given by multiplying the c.p.m. in isolated glycogen (after correction for the sodium sulphate effect and the background) by the number of micromoles of glucose added and dividing this figure by the c.p.m. in the labelled glucose added whose radioactivity was determined under the same conditions but in the absence of sodium sulphate.

Measurement of oxygen uptake: Oxygen uptake was measured as described by Nigam (1966). Samples were pre-incubated in the presence or absence of epinephrine and glucose solution was contained in the side arm. Measurements of oxygen consumption were made before and after the addition of glucose.

Glucose determination: Glucose was determined in the supernatant obtained after the reaction mixture had been boiled at 100°C for 1 min. Sugar phosphates were removed by Ba(OH)₂-ZnSO₄ treatment and glucose was estimated according to Ashwell (1957).

Lactic acid determination: Lactic acid was determined in the supernatant of heat inactivated reaction mixtures by the chemical procedure of Barker (1957). In a few instances, determination of lactic acid by the chemical method was checked with the enzymatic procedure of Hohorst (1963). Differences of ±4% were observed between the two methods.

Determination of glucose 6-phosphate, fructose diphosphate, AMP and ATP: Glucose
6-phosphate and ATP were determined according to Nigam (1966), AMP according to Adams (1963) and fructose diphosphate as described by Cooper et al. (1958).

RESULTS

Effect of varying concentrations of epinephrine on the glucose metabolism in Novikoff ascites-hepatoma cells

Fig. 1 shows the amount of glycogen synthesised, glucose utilized and lactate produced by Novikoff ascites-hepatoma cells incubated with glucose in the presence of epinephrine bitartrate solutions of different concentrations. Ascorbic acid (1 mM) was present in the reaction mixtures in order to prevent oxidation of epinephrine. It is apparent from Fig. 1 that there is no major effect of varying epinephrine concentrations on the three measured parameters. However, minor effects can be noted. For example, at lower concentration (2 x 10^{-5} M), epinephrine exerted a slight stimulatory effect on glycogen synthesis and a slight inhibitory effect on lactate production. Further increase in concentration abolished this response and at higher concentration (1 mM), both glycogen synthesis and glycolysis were slightly depressed. In consequence, glucose utilisation apparently decreased as the epinephrine concentration was increased.

![Graph showing glycogen formation, lactate production and glucose utilisation](image_url)

**Fig. 1.**

Glycogen formation, lactate production and glucose utilisation by intact Novikoff ascites-hepatoma cells in the presence of varying concentrations of epinephrine. Experimental conditions are described under Materials and Methods. The values are averages of those in three separate experiments.
Effect of epinephrine on respiration and levels of glucose 6-phosphate, fructose diphosphate and adenine nucleotides in Novikoff ascites-hepatoma cells

Novikoff ascites hepatoma cells have been shown in earlier studies (Nigam 1966) to have no Crabtree effect. In these experiments, a slight (approx. 4%) inhibition of respiration on glucose addition was observed (Table 1). Epinephrine (2 x 10⁻⁵ M) caused a further decrease (3%) in oxygen consumption when added alone or when added to cells containing glucose. ATP levels in cells incubated under these conditions also showed minor variations. The levels of glucose 6-phosphate and fructose diphosphate demonstrated that glucose 6-phosphate was increased and fructose diphosphate decreased when epinephrine was present with cells incubated in the presence of glucose. Inspite of small variations in the concentration of these metabolites, an increase in ATP and a decrease AMP concentrations (Wu 1964), and higher glucose 6-phosphate to fructose diphosphate ratio (Wu 1964) on epinephrine addition were indicative of an inhibition of phosphofructokinase.

Table 1.
Effect of epinephrine on respiration and on the concentration of glucose 6-phosphate fructose diphosphate and adenine nucleotides in Novikoff ascites-hepatoma cells.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Oxygen consumed/10 min (μl)</th>
<th>AMP (μmoles/ml of packed cells)</th>
<th>ATP (μmoles/ml of packed cells)</th>
<th>G-6-P (μmoles/ml of reaction mixture)</th>
<th>FDP (μmoles/ml of reaction mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3</td>
<td>1  2  1  2</td>
<td>1  2  1  2</td>
<td>1  2  1  2</td>
<td>1  2  1  2</td>
</tr>
<tr>
<td>Additions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA alone</td>
<td>38.5 41 38</td>
<td>– – 1.4 1.6</td>
<td>– – 1.3 1.4</td>
<td>0.06 0.04 0.04 0.02</td>
<td></td>
</tr>
<tr>
<td>AA + glucose</td>
<td>37.0 39.5 36.8</td>
<td>0.19 0.17 1.3 1.4</td>
<td>– – 1.3 1.4</td>
<td>0.06 0.04 0.04 0.02</td>
<td></td>
</tr>
<tr>
<td>AA + epinephrine</td>
<td>36.2 40.0 37.0</td>
<td>– – 1.2 1.25</td>
<td>– – 1.3 1.4</td>
<td>0.07 0.07 0.03 0.01</td>
<td></td>
</tr>
<tr>
<td>AA + glucose + epinephrine</td>
<td>35.6 38.0 35.5</td>
<td>0.15 0.15 1.6 1.9</td>
<td>– – 1.3 1.4</td>
<td>0.07 0.07 0.03 0.01</td>
<td></td>
</tr>
</tbody>
</table>

For studies on respiration, 2.5 ml tumour cell suspension were added to the main compartment of Warburg vessels. The cell suspensions contained either 1 mM ascorbic acid (AA) or 1 mM ascorbic acid and 2 x 10⁻⁵ M epinephrine bitartrate (E). Glucose solution (0.5 ml containing 30 μmoles glucose) was contained in the side arm. After equilibration for 10 min, measurements of O₂ uptake were made before and after the addition of glucose. In separate experiments, concentrations of AMP, ATP, glucose 6-phosphate (G 6-P) and fructose diphosphate (FDP) were determined in neutral perchloric acid extracts after incubation of tumour cell suspension in 20-ml beakers in a Dubnoff metabolic shaker at 37°C (see Materials and Methods) with and without glucose and in the presence of ascorbic acid alone and ascorbic acid + epinephrine bitartrate.
Glycogen and lactate formation from glycogen precursors by fresh and aged cells in the presence and absence of epinephrine

Recent studies from this laboratory have shown that glucose phosphates and UDPglucose enter tumour cells if they have been aged for 18-24 h at 0-5°C (Nigam 1967a,b). The effect of epinephrine on specific enzymes of glycogen pathway can thereby be ascertained when various precursors of glycogen are incubated with intact aged tumour cells in the presence of epinephrine. Table 2 shows the amount of glycogen synthesised and lactate produced from glucose-6-phosphate, glucose 1-phosphate and UDPglucose by both freshly prepared and aged cells in the presence and absence of $2 \times 10^{-5}$ M epinephrine. Epinephrine stimulated glycogen synthesis slightly from both glucose and glucose 1-phosphate. On the other hand, glycogen synthesis from glucose 6-phosphate was slightly decreased. When UDPglucose was used as the glycogen precursor, epinephrine stimulated glycogen synthesis both in the absence and presence of glucose 6-phosphate. The extent of increase varied from 5 to 25 per cent. The amount of lactate produced from various substrates in aged cells was high, and no major differences due to epinephrine addition could be observed.

Table 2.
Glycogen and lactate formation from various substrates by tumour cells in the presence and absence of epinephrine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
<th>Glycogen formed* (as μmoles glucose)</th>
<th>Lactate produced*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fresh cells</td>
<td>Aged cells</td>
</tr>
<tr>
<td>Glucose</td>
<td>AA</td>
<td>1.8 ± .3</td>
<td>0.80±.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>AA + E</td>
<td>1.9 ± .3</td>
<td>0.85±.12</td>
</tr>
<tr>
<td>G-6-P</td>
<td>AA</td>
<td>0.07±.01</td>
<td>0.12±.01</td>
</tr>
<tr>
<td>G-6-P</td>
<td>AA + E</td>
<td>0.05±.015</td>
<td>0.10±.02</td>
</tr>
<tr>
<td>G-1-P</td>
<td>AA</td>
<td>0.10±.00</td>
<td>0.22±.12</td>
</tr>
<tr>
<td>G-1-P</td>
<td>AA + E</td>
<td>0.15±.02</td>
<td>0.25±.02</td>
</tr>
<tr>
<td>UDPG</td>
<td>AA</td>
<td>0.78±.1</td>
<td>1.3±.2</td>
</tr>
<tr>
<td>UDPG</td>
<td>AA + E</td>
<td>0.88±.2</td>
<td>1.5±.2</td>
</tr>
<tr>
<td>UDPG</td>
<td>AA + G-6-P</td>
<td>1.5±.2</td>
<td>2.1±.4</td>
</tr>
<tr>
<td>UDPG</td>
<td>AA + G-6-P+E</td>
<td>1.9±.3</td>
<td>2.2±.3</td>
</tr>
</tbody>
</table>

The composition of the digest is described under Materials and Methods. It contained 5 μmoles labelled substrate (approx. 50 000 counts/min) and either 1 mM ascorbic acid (AA) or 1 mM ascorbic acid and $2 \times 10^{-5}$ M epinephrine bitartrate (E). With UDP-glucose as substrate, 5 μmoles unlabelled glucose 6-phosphate was also added in certain experiments. The volume of the digest was kept 1 ml in each case. The values are means±S.D. of those obtained in three experiments.

* μmoles/ml digest/15 min.

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Action of epinephrine on glycogen breakdown

Unlike the glycogenolytic action of epinephrine in muscle, tumour cells were insusceptible to its action. Thus, cells in which glycogen has been deposited by prior incubation with glucose-1-\(^{14}\)C (and free glucose removed by washing the cells), showed no increased disappearance of label from glycogen on further incubation in the presence of epinephrine (Table 3). On the contrary at higher concentrations (1 mM) of epinephrine there was inhibition in the removal of glycogen label as compared to the control (not shown). Addition of dinitrophenol accomplished rapid breakdown of glycogen, due to increase in AMP concentration required for activation of phosphorylase \(b\) (Nigam 1967a). Cyclic AMP (1 mM) and 5'-AMP (1 mM) also decreased glycogen label but were not as effective as DNP (Table 3).

DISCUSSION

Ineffectiveness of epinephrine to induce major alteration in glucose and glycogen metabolism of Novikoff ascites-hepatoma cells provides an instance where neoplastic transformation results in the abolition of hormonal response. The absence of glycogenolytic action of epinephrine in Novikoff ascites-hepatoma

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Min of incubation</th>
<th>Counts/min in isolated glycogen</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. No.</td>
<td>AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA alone</td>
<td>AA + E</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12,000</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10,200</td>
<td>11,800</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>19,200</td>
<td>19,200</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16,000</td>
<td>18,100</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>11,900</td>
<td>11,900</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>11,200</td>
<td>11,500</td>
</tr>
</tbody>
</table>

Tumour cell suspensions were incubated with 10 \(\mu\)moles \(^{14}\)C glucose (50,000 counts/min) for 10–20 min in 20 ml beakers as described under Materials and Methods, to deposit labelled glycogen in the cells. The reaction mixtures were then combined, centrifuged and the sedimented cells washed with cold Tris-incubation medium to free them of \(^{14}\)C glucose. They were then resuspended in the medium and 1 ml suspension were incubated with either 1 mM ascorbic acid (AA) or 1 mM ascorbic acid and \(2 \times 10^{-5} \) M epinephrine bitartrate (E) and with 0.25 M dinitrophenol (DNP), 1 mM AMP and 1 mM cyclic AMP under conditions similar to those described under Materials and Methods. Glycogen was isolated before and after incubation and counted for its \(^{14}\)C.
cells, which contain both phosphorylase and phosphorylase kinase (Nigam, unpublished), indicates that epinephrine does not bring about glycogen breakdown in epinephrine-sensitive tissues by acting directly on these enzymes. Neither does it have the capacity to induce ATP breakdown and increase AMP concentration so that activation of phosphorylase \( b \) can be obtained. It is likely, therefore, that among many proteins deleted in tumour tissues, the receptor responsible for epinephrine action in liver may have suffered a loss during neoplastic transformation.

Low concentrations of epinephrine \((1 \times 10^{-7})\), normally effective in eliciting physiological response in muscle, had no effect on glycogen metabolism in this tumour. At high concentration \((2 \times 10^{-5})\) epinephrine exerted a small but reproducible effect of activating glycogen synthesis and inhibiting both glycogen breakdown and glycogenolysis. Such effect has not been reported for normal tissues responsive to the action of epinephrine. However, Mayer et al. (1967) have observed that epinephrine infusion to the rat \((2.5 \mu g/kg/min)\) results initially in an increase in the activities of phosphorylase \( a \) and the glucose 6-phosphate independent form of UDPglucose-\( \alpha \)-glucan glucosyltransferase. These changes are subsequently reversed. It is possible that in Novikoff ascites-hepatoma cells the initial phase of epinephrine action may be prolonged and the activation of phosphorylase does not surpass the increase in the activity of UDPglucose-\( \alpha \)-glucan glucosyltransferase as it happens in case of rat heart (Mayer et al. 1967). Indeed, determinations of phosphorylase \( a \) and glucose 6-phosphate independent form of UDPglucose-\( \alpha \)-glucan glucosyltransferase activities in tumour cell homogenates before and after incubation with epinephrine show only minor variations in the activities of the two enzymes due to epinephrine. Since the changes induced by epinephrine on glycogen metabolism of tumour cells are themselves very small, it is not unusual that significant changes in activities of these enzymes were not detected.

The observed data on glucose 6-phosphate and AMP concentration in the tumour cell would normally support that conditions after epinephrine addition are favourable to the activation of UDPglucose-\( \alpha \)-glucan glucosyltransferase and inhibition of phosphorylase (Fridland & Nigam 1965), except for the fact that epinephrine increases glycogen synthesis from UDPglucose even in the presence of glucose 6-phosphate (Table 2). Karpatkin et al. (1964) have also observed that although epinephrine induces loss in glycogen and increase in glucose 6-phosphate concentration in frog muscle, there is no activation of UDPglucose-\( \alpha \)-glucan glucosyltransferase by accumulated glucose 6-phosphate so that glycogen could be rebuilt from its precursors. Indeed Craig & Larner (1964) have shown that epinephrine mediated decrease in UDP-glucose-\( \alpha \)-glucan glucosyltransferase activity of rat diaphragm results from a loss of both the glucose 6-phosphate dependent and independent forms of the enzyme.

The effect of epinephrine on phosphorylase has been shown to be mediated
by cyclic AMP (Robinson et al. 1967). Cyclic AMP also diminishes the glucose 6-phosphate dependent and independent activities of UDPglucose-α-glucan glucosyltransferase (Belocopitow 1961). However, in case of Novikoff ascites-hepatoma cells exogenous addition of cyclic AMP has no effect on glycogen synthesis from glucose by the tumour cells. Cyclic AMP, nevertheless, does enhance glycogen breakdown although it is not as effective as AMP. Further, the effect of AMP on glycogen degradation is considerably smaller than that of DNP suggesting that the nucleotides have either poor permeability or they are transformed into ATP once they enter the cell. In any case the response of cyclic AMP and epinephrine differs from each other to warrant drawing a general conclusion that cyclic AMP acts universally as a messenger of epinephrine action.

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