A Study of the Crabtree Effect in Novikoff Ascites-Hepatoma Cells

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Novikoff ascites-hepatoma cells show no Crabtree effect on the addition of glucose to tumour-cell suspensions, and convert a significant part of the added glucose into glycogen. Treatment of the cells with 2-deoxyglucose or glucose in the presence of iodoacetate inhibits respiration and decreases glycogen synthesis from glucose. Short-term experiments indicate a slight inhibition of glucose uptake for a brief period, due either to ATP accumulation in the mitochondria or to glucose 6-phosphate-mediated inhibition of hexokinase. Utilization of glucose metabolites and ATP for glycogen synthesis appears to remove inhibition of glucose uptake, and perhaps accounts for the absence of respiratory inhibition, by relieving a deficiency of ADP for the mitochondria. Decreased respiration in the presence of 2-deoxyglucose or glucose in the presence of iodoacetate could be due to the change in mitochondrial structure or permeability, caused by the significant loss of adenine nucleotides.

The discovery by Crabtree (1929) that respiration of tumour cells is inhibited by metabolizable sugars remains an unexplained phenomenon of metabolic control. The findings that Crabtree effects occur with glucose even in the presence of iodoacetic acid (Wu & Racker, 1959b) and that 2-deoxyglucose elicits a Crabtree effect (Ibsen, Coe & McKee, 1958) suggested that the presence of a sugar capable of undergoing phosphorylation is the only requirement for the inhibition of respiration. The short-term studies by Hess & Chance (1961) with intact Ehrlich ascites-tumour cells and by Sauer (1964) with isolated tumour mitochondria revealed that initial glucose phosphorylation results in intramitochondrial ATP accumulation followed by inhibition of glucose phosphorylation, due either to non-availability of ATP or to glucose 6-phosphate-mediated inhibition of hexokinase, and consequently the suppression of respiration due to ADP deficiency.

A previous communication from this Laboratory showed that Novikoff ascites-hepatoma cells utilize glucose to form glycogen mainly at the expense of aerobically produced ATP (Nigam, 1965). These cells therefore afford the possibility of testing whether utilization of mitochondrial ATP during glucose metabolism can overcome a deficiency of mitochondrial ADP so as to show little or no Crabtree effect in this tumour. The present paper deals with the study of oxygen consumption during glucose utilization and variations in the concentrations of essential metabolites and end

products. 2-Deoxyglucose and glucose with iodoacetic acid were used as control substances, the former being a substance that does not form glycogen. The results obtained in this study suggest, in general, that glycogen synthesis can eliminate the Crabtree effect and that the mediation of the effect in other tumours is probably due to a deficiency of mitochondrial ADP produced by a lack of mitochondrial ATP utilization. The reasons for the Crabtree effect with 2-deoxyglucose and glucose in the presence of iodoacetate appear to be different.

MATERIALS AND METHODS

Chemicals. Most of the non-labelled chemicals used in the present study were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. [^{14}C]Glucose was obtained from Merck and Co., Montreal, Canada.

Preparation of tumour cells. Novikoff and Ehrlich ascites-tumour cells were grown and prepared as described by Nigam (1962a) and by Wu & Racker (1959a) respectively.

Glucose determination. Glucose was determined in the supernatant obtained from incubation mixtures after the addition of HClO_4 , removal of perchlorate with KOH in the cold and removal of sugar phosphates by $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ treatment, as described by Ashwell (1957).

Lactic acid determination. Lactate was determined in neutral HClO_4 extracts according to Barker (1957).

Incorporation of [^{14}C]glucose into glycogen. Glycogen formation was measured by its isolation and estimation of its ^{14}C as described by Nigam & Fridland (1964).

Determination of glucose 6-phosphate and ATP. Glucose 6-phosphate and ATP were measured in the same incubation

mixture, which contained (in 3 ml.): glycylglycine buffer, pH 7.4, 150 μ moles; $MgCl_2$, 15 μ moles; EDTA, 10 μ moles; glucose, 30 μ moles; $NADP^+$, 3 μ moles; glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 1 unit. The increase in $E_{340\text{ m}\mu}$ after the addition of the glucose 6-phosphate gave the amount of glucose 6-phosphate. The additional increase in $E_{340\text{ m}\mu}$ after the addition of hexokinase (EC 2.7.1.1) gave the amount of ATP in the digest. The procedure was similar to that used by Wu (1964).

Determination of ADP. ADP was estimated with crystalline pyruvate kinase (EC 2.7.1.40) and the trisodium salt of phosphoenolpyruvate, with lactate dehydrogenase (EC 1.1.1.27) and NADH as the indicator system, in an incubation mixture similar to that used for the estimation of C_3 intermediates but containing no ADP (Nigam, 1962b). The decrease in $E_{340\text{ m}\mu}$ after the addition of pyruvate kinase gave the amount of ADP; trace amounts of pyruvate were removed as lactic acid by the addition of lactate dehydrogenase and NADH before the addition of pyruvate kinase.

Measurement of oxygen uptake. The rate of respiration in cell suspensions was measured by the conventional Warburg technique (Umbreit, Burris & Stauffer, 1964), after a preincubation period of 10 min. Sugar solution was tipped from the side arm into the main chamber after the preincubation. The fluid volume was maintained between 2.5 and 3.0 ml. in various experiments. Carbon dioxide was absorbed by 0.2 ml. of 10% (w/v) KOH in the centre well with a Whatman no. 1 filter-paper strip inserted in it.

Incubation techniques. Ascites-tumour-cell suspensions containing approx. 2–3 ml. of packed cells/10 ml. were prepared by using the tris incubation medium described by Wu & Racker (1959a). A 2–2.5 ml. portion of this suspension was used for the measurement of respiration. In short-term experiments, the suspension (0.9 ml.) was transferred to 20 ml. beakers, preincubated for 5 min. in a Dubnoff metabolic shaker and sugar solution (0.1 ml.) was added. At various time-intervals 0.5 ml. of 0.6 N- $HClO_4$ was added to the digests. The supernatant obtained after the removal

of protein was neutralized in the cold with KOH, and $KClO_4$ was removed. The supernatant was used for the estimation of glucose, glucose 6-phosphate, ADP and ATP.

RESULTS

Oxygen uptake, glycolysis and glycogen synthesis by Ehrlich and Novikoff ascites-tumour cells. Table 1 shows the rate of oxygen uptake in the absence and presence of glucose by Ehrlich and Novikoff ascites-tumour cells. Data are also presented on the amount of lactate produced and the glycogen formed by the two tumours during a 30 min. incubation. The addition of glucose to Novikoff hepatoma cells appears to have little effect on their respiratory rate, whereas Ehrlich ascites-tumour cells undergo the typical respiratory inhibition commonly observed with this tumour. The other difference between the two tumours is that Novikoff ascites-hepatoma cells can metabolize glucose to form significant amounts of glycogen as well as lactic acid, whereas the Ehrlich ascites tumour cannot form glycogen, although lactate is produced.

Oxygen uptake by Novikoff ascites-tumour cells in the presence of 2-deoxyglucose and glucose with iodoacetic acid. Incubation of Novikoff ascites-tumour cells with 2-deoxyglucose resulted in a decrease of respiration that increased with time (Table 2). Iodoacetic acid added alone inhibited respiration of the tumour cells slightly. However, the addition of glucose to iodoacetate-treated cells gave respiration that followed a pattern similar to that observed for 2-deoxyglucose. Glycogen synthesis was also suppressed in both cases. The addition of [^{14}C]glucose to cells that had been

Table 1. *Rate of oxygen uptake and the amount of lactate and glycogen formed in the presence and absence of glucose by Novikoff and Ehrlich ascites-tumour cells*

Tumour-cell suspension (2.0 ml.) containing 0.2–0.25 ml. of packed cells was transferred to the main compartment of a Warburg flask. After 10 min. of equilibration, 0.5 ml. of glucose solution (20 μ moles, 10^5 counts/min.) was transferred from the side arm. Oxygen uptake was measured every 10 min. and the glycogen and lactate formed were estimated after 30 min. of incubation. The values are averages of three experiments.

Time	O ₂ uptake (μ l./flask)			
	Novikoff tumour		Ehrlich tumour	
	Glucose absent	Glucose present	Glucose absent	Glucose present
First 10 min.	19.5	19.8	27.2	17.6
Second 10 min.	22.8	23.1	27.5	15.8
Third 10 min.	22.8	23.2	27.6	15.8
Glycogen formed*	0.0	7.2	0.0	0.2
Lactate formed†	0.4	6.8	0.6	8.9

* μ moles of glucose equivalent of glycogen formed/30 min./reaction flask.

† μ moles of lactate formed/30 min./reaction flask.

Table 2. *Effect of 2-deoxyglucose and glucose with iodoacetate on oxygen uptake by Novikoff ascites-hepatoma cells*

Experimental details are the same as described in Table 1. The side arm contained the incubation medium (0.5 ml.) alone, or 0.5 ml. of a solution of iodoacetate (2.5 mM) in the medium alone, or with glucose (20 μ moles), or 2-deoxyglucose (20 μ moles) solution alone. The solutions from the side arm were tipped after 10 min. of equilibration. The values are averages of two experiments.

Time	Treatment ...	O ₂ uptake (μ l./flask)			
		None	Iodoacetate	Iodoacetate + glucose	2-Deoxy-glucose
First 10 min.		19.5	18.5	16.5	18.7
Second 10 min.		22.8	17.3	12.8	15.4
Third 10 min.		22.8	17.0	11.0	12.3

Table 3. *Glycogen synthesis by Novikoff ascites-hepatoma cells pretreated with iodoacetate, glucose and iodoacetate and 2-deoxyglucose*

Tumour-cell suspension (0.9 ml.) containing 0.2 ml. of packed cells was preincubated with iodoacetate (0.5 μ mole), with glucose (10 μ moles) and iodoacetate (0.5 μ mole), or with 2-deoxyglucose (10 μ moles). Additions were made by dissolving the substances in 0.05 ml. of medium. After 10 min. of preincubation, [¹⁴C]glucose (10 μ moles, 10⁵ counts/min.) solution (0.05 ml.) was added and digests were incubated for 30 min. In the case of prior treatment with glucose and iodoacetic acid, the amount of glucose added was small (but contained the same amount of ¹⁴C), so that the amount of glucose before the start of the experiment was 10 μ moles.

Treatment	Glycogen synthesis (μ moles of glucose equiv. of glycogen formed/30 min./0.2 ml. of packed cells)
None	6.5
Iodoacetate	1.1
Iodoacetate + glucose	0.4
2-Deoxyglucose	0.1

treated with 2-deoxyglucose or with iodoacetic acid resulted in complete inhibition of glycogen formation in the former and a partial inhibition in the latter case (Table 3).

Glucose uptake and changes in the concentrations of glucose 6-phosphate, ATP and ADP. A short-term experiment was designed to see the immediate changes that took place in the concentrations of ADP, ATP and glucose 6-phosphate after the addition of glucose to the tumour cells. Fig. 1 shows that the added glucose is rapidly utilized; the rate of utilization falls between $\frac{1}{2}$ and 2 min. and then continues after 2 min. The concentration of ATP in this tumour is low; it falls after the addition of glucose and does not return to its original value. The concentration of ADP rises

Fig. 1. Time-course of glucose utilization ($\square$), and variations in the concentrations of ATP ($\blacksquare$), ADP ($\circ$) and glucose 6-phosphate ($\bullet$), after the addition of glucose (10 μ moles) solution (0.1 ml.) to a suspension (0.9 ml.) of Novikoff ascites-tumour cells containing 0.2 ml. of packed cells. Experimental details are given in the text. The plots are representative of three separate experiments.

slightly at $\frac{1}{2}$ min. and continues to be the same thereafter. Glucose 6-phosphate shows an increase and a fall after 1 min. The lower value is maintained thereafter. The sequence of changes that occur have some resemblance to the observations by Hess & Chance (1961), though the changes are not so marked. Further, the recovery in glucose utilization after the partial inhibitions in the early stages occurs after the establishment of relatively lower concentrations of ATP and glucose 6-phosphate and a proportionately higher concentration of ADP.

Fig. 2. Variation in the concentrations of ATP (----) and P_i (—) on the addition of a solution (0.1 ml.) of 10 μ moles of glucose (●) or 2-deoxyglucose (○) to a suspension (0.9 ml.) of Novikoff ascites-tumour cells containing 0.2 ml. of packed cells. The concentrations of ATP and P_i were the same on the addition of glucose to a tumour-cell suspension pretreated with 0.5 mM-iodoacetate as those obtained on the addition of 2-deoxyglucose. The values are averages of those in two experiments.

Variation in the concentrations of ATP and P_i during incubation with glucose, 2-deoxyglucose and glucose plus iodoacetic acid. Fig. 2 shows a comparative study of changes in ATP and P_i when tumour cells are incubated with glucose, 2-deoxyglucose and glucose plus iodoacetic acid. There is a remarkable similarity between the effect of treatment with 2-deoxyglucose and that obtained with glucose plus iodoacetate. The P_i concentration decreases to about half its original value and stays low, whereas the concentration of ATP falls to a negligible value within the first minute of incubation, and shows no increase on further incubation. In this respect the effect of the addition of glucose differs significantly. Although glucose brings about a decrease in P_i concentration, the P_i concentration slowly recovers to about 70% of the initial value. Thus Novikoff ascites-hepatoma cells differ from Ehrlich ascites-tumour cells, in which P_i concentrations continue to be lower after the addition of glucose (Wu & Racker, 1959b).

DISCUSSION

Two types of Crabtree effects have been observed in tumour cells. One, measured just after the addition of glucose, is called the 'short-term' Crabtree effect; the other, obtained after 30–60 min. of incubation, is referred to as the 'long-term' or 'classical' Crabtree effect. The short-term effect is characterized by an initial rapid glucose utilization, giving increased respiration, followed by an intense inhibition of both respiration and glucose uptake

for a brief period (Hess & Chance, 1961). The inhibitions are relieved by uncouplers of oxidative phosphorylation. The short-term Crabtree effect has been successfully reproduced in isolated mitochondria by Sauer (1964). Hess & Chance (1961) suggest that a change in mitochondrial permeability occurs owing to ATP accumulation in the mitochondria, and that this is responsible for lack of ATP utilization by glucose and a lack of ADP availability for respiration. The experiments of Sauer (1964), however, show a glucose 6-phosphate-mediated inhibition of hexokinase, since the addition of ATP does not relieve respiratory inhibition. The first hypothesis lacks experimental support for a change in mitochondrial permeability; Sauer's (1964) work was done with isolated mitochondria and any respiratory inhibition of intact cells had to inhibit not only the hexokinase but also other ADP-producing systems that operate in the absence of added glucose.

The long-term Crabtree effect is characterized by a varying degree (10–50%) of respiratory inhibition in the presence of a sugar phosphorylated in the presence of hexokinase. It is also obtained with 2-deoxyglucose and with glucose in the presence of iodoacetate. One of the features of the classical Crabtree effect is that the ATP lost by inhibition of respiration in the presence of metabolizable sugar is replaced by an exact amount produced through glycolysis (Quastel & Bickis, 1959). The lack of ATP production from 2-deoxyglucose utilization or from glucose when iodoacetate is present limits the acceptability of the Crabtree effect obtained under these conditions as a classical Crabtree effect. Further, under both conditions a release of intracellular nucleotides from the cells into the medium in the form of nucleosides occurs (Wu & Racker, 1959a; McComb & Yushok, 1964).

The results presented above show the absence of a long-term Crabtree effect from Novikoff ascites-hepatoma cells. Since a large part of added glucose is utilized in glycogen formation, this tumour utilizes ATP besides producing it through glycolysis. The preferential requirement for aerobic ATP for glycogen synthesis suggest an availability of mitochondrial ADP for respiration if ADP is presumed to be rate-limiting for respiration. Thus metabolic reactions of glucose undergone in Novikoff ascites-hepatoma cells differ markedly from those observed in the Ehrlich ascites-tumour cells. The latter have the mechanisms to produce ATP from glucose, but cannot utilize glucose or glucose metabolites in ATP-consuming reactions. It is the imbalance in ATP utilization that results in the decrease of ATP production through respiration.

In an attempt to study the role of glucose 6-phosphate, ATP and ADP in the Crabtree effect, these factors were analysed in short-term experi-

ments. Although the initial reaction resulted in an increase in the glucose 6-phosphate concentration, this soon fell to one-half its maximum value and stayed lower. The ADP concentration rose after 30 sec. and stayed higher, but the ATP concentration fell and increased only slightly during further incubation. The conditions after 2–3 min. are such that hexokinase and phosphofructokinase inhibition cannot be mediated with any efficiency and respiration can hardly be inhibited, owing to ADP deficiency. These findings differ markedly from those reported by Wu (1965) for Ehrlich ascites-tumour cells.

However, there appears to be a similarity between the two types of tumour cells insofar as the initial period is concerned. There is a slight lag in glucose utilization when the concentration of glucose 6-phosphate is high. These results make it difficult to differentiate between glucose 6-phosphate-mediated inhibition of hexokinase and lack of ATP availability for hexokinase due to its accumulation in the mitochondria. In any case the ATP or glucose 6-phosphate utilization for glycogen synthesis relieves the inhibition, and glucose utilization and respiration continue un-suppressed.

The balance of energy that exists in the classical Crabtree effect is disrupted in Novikoff ascites-hepatoma cells. Since glycolysis proceeds in the absence of suppressed respiration, more ATP is produced in the presence than in the absence of glucose. Most of the aerobic energy appears to be utilized in glucose polymerization. Although it is not known how the energy balance is controlled in tumour cells during the Crabtree effect, the following explanation is suggested. If it is presumed that ADP formed by ATP breakdown is shared between the glycolytic system and the respiratory chain according to the relative affinities of their transphosphorylating systems for ADP, whereas the rate of ADP breakdown remains the same both in the absence or presence of glucose, then the total ATP production will remain unaltered, though part will be derived through glycolysis and the rest from respiration. Conversely, when aerobic ATP production is abolished by anaerobiosis, more ADP would be available to the glycolytic system (as shown by the observed high ratio of ADP to ATP in anaerobic rather than aerobic conditions), thereby increasing the rate of glycolysis. This consideration satisfies both Pasteur and Crabtree effects. Since these two effects are essentially the reverse of one another, they may have a common basis. In Novikoff ascites-hepatoma cells it appears that the rate of aerobic ATP breakdown increases during glycogen synthesis; it responds by a maintenance of respiration at uninhibited rates.

2-Deoxyglucose-mediated inhibition provides intracellular conditions different from those ob-

tained after glucose addition. Since the addition of glucose to cells pretreated with iodoacetate provides identical conditions, respiratory inhibition mediated by 2-deoxyglucose can be ascribed, not to the structure of the molecule, but more appropriately to its trapping action of the terminal phosphate of ATP in the hexokinase reaction and the inability of its metabolites to transphosphorylate ADP. Under both conditions a fall in the concentrations of ATP and P_i follows respiratory inhibition. The initial events bring about a fast breakdown of ATP that is not matched by its resynthesis; this results in ADP availability to the ADP-degrading enzymes, namely adenylate kinase, adenylate deaminase and 5'-nucleotidase. All these enzymes have been found in the extracts of Novikoff ascites hepatoma. Since the concentration of ATP falls steeply and remains low, the ATP-depleted mitochondria in the cell may tend to swell and thereby show loss of their respiratory activity. In my experiments it has generally been observed that incubation of the Novikoff ascites-hepatoma cells under these conditions results in their swelling. Whether swelling of the mitochondria in the intact cells also occurs remains to be established, although loss of ATP and its role in mitochondrial contraction (Lehninger, 1962) suggest it as a clear possibility.

The decrease in 2-deoxyglucose phosphorylation soon after a rapid initial reaction (McComb & Yushok, 1964; Letnansky, 1964) suggests that the fall in the concentration of ATP cannot support further 2-deoxyglucose phosphorylation. Similarly loss of glycogen synthesis after prior treatment with 2-deoxyglucose may be due to a restricted ATP availability for reactions mediating glucose polymerization. It is likely that ATP produced through diminished respiration reacts directly with AMP in the adenylate-kinase reaction in preference to its reaction with hexokinase and 2-deoxyglucose. Colowick (1955) has also suggested that ATP produced by the mitochondria might react with AMP within the particles preferentially to reaction with hexokinase and glucose present externally.

The decrease in respiratory rate after the loss of adenine nucleotides is not restricted to hexokinase-sensitive sugars alone. Thus 3'-deoxyadenosine has been shown to be phosphorylated to its polyphosphates by Ehrlich ascites-tumour cells, with concomitant loss of adenine nucleotides as inosine; depletion of the nucleotide pool follows respiratory inhibition (Overgaard-Hansen, 1966). The respiratory inhibition mediated by 2-deoxyglucose or glucose in the presence of iodoacetate should therefore be regarded as a special case, perhaps only distantly related to the classical Crabtree effect. It does not appear to be caused by ATP accumulation in the mitochondria, hexokinase inhibition due to product accumulation, or ADP deficiency. The

fall in concentration of P_i is not large, nor does externally added P_i remove the inhibition (Yushok, 1964), to make P_i as the main rate-limiting factor for the respiration.

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REFERENCES

- Ashwell, G. (1957). In *Methods in Enzymology*, vol. 3, p. 85. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Barker, S. B. (1957). In *Methods in Enzymology*, vol. 3, p. 241. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Colowick, S. P. (1955). In *Methods in Enzymology*, vol. 2, p. 604. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Crabtree, H. G. (1929). *Biochem. J.* **23**, 536.
- Hess, B. & Chance, B. (1961). *J. biol. Chem.* **236**, 239.
- Ibsen, K. H., Coe, E. L. & McKee, R. W. (1958). *Biochim. biophys. Acta*, **30**, 384.
- Lehninger, A. L. (1962). *Physiol. Rev.* **32**, 467.
- Letnansky, K. (1964). *Biochim. biophys. Acta*, **87**, 17.
- McComb, R. B. & Yushok, W. D. (1964). *Cancer Res.* **24**, 193.
- Nigam, V. N. (1962a). *Nature, Lond.*, **196**, 478.
- Nigam, V. N. (1962b). *Canad. J. Biochem. Physiol.* **40**, 836.
- Nigam, V. N. (1965). *Biochem. J.* **95**, 1c.
- Nigam, V. N. & Fridland, A. (1964). *Biochem. J.* **92**, 3c.
- Overgaard-Hansen, K. (1966). *Biochem. J.* (in the Press).
- Quastel, J. H. & Bickis, I. J. (1959). *Nature, Lond.*, **183**, 281.
- Sauer, L. A. (1964). *Biochem. biophys. Res. Commun.* **17**, 294.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964). *Manometric Techniques*, 4th ed., pp. 7-17. Minneapolis: Burgess Publishing Co.
- Wu, R. (1964). *Biochem. biophys. Res. Commun.* **14**, 79.
- Wu, R. (1965). *J. biol. Chem.* **240**, 2827.
- Wu, R. & Racker, E. (1959a). *J. biol. Chem.* **234**, 1029.
- Wu, R. & Racker, E. (1959b). *J. biol. Chem.* **234**, 1036.
- Yushok, W. D. (1964). *Cancer Res.* **24**, 187.