RELATION OF ENERGY PROCESSES TO THE INCORPORATION OF AMINO ACIDS INTO PROTEINS OF THE EHRlich ASCITES CARCINOMA*

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Considerable evidence has accumulated that the incorporation of amino acids into protein requires the participation of an external source of energy (1). The requirement for this energy source has been linked to that made available by oxidation. Anaerobic conditions have been reported to produce complete or variable inhibition of incorporation in several tissues (1). The possible participation of energy contributed by anaerobic glycolysis has not been fully investigated. In this communication it is shown that glycolysis can supply energy for the incorporation of several amino acids into the protein of the Ehrlich ascites carcinoma.

The nature of this energy requirement has been the subject of considerable speculation, and various postulations involving phosphorylated amino acids (2), transpeptidation reactions (3), and activated template mechanisms (4) have been suggested. An attempt is made here to define more clearly the nature of the activation process. To this end the extent of incorporation into protein of amino acids, both singly and in groups, was determined under conditions of a suboptimal energy supply brought about by the addition of 2,4-dinitrophenol.

**Methods**

The procedures employed were those described previously (5), with variations in the contents of the Warburg flasks and time of incubation as indicated for individual experiments. The radioactive amino acids were also the same with the addition of dl-methionine-2-C\(^{14}\) (2.1 μc. per mg.), which was obtained from Tracerlab, Inc., Boston. Non-radioactive dl-methionine was obtained from the Nutritional Biochemicals Corporation, Cleveland. The 2,4-dinitrophenol (DNP) was an Eastman Kodak product which was recrystallized twice from water. When oxygen uptake was measured, air was used in the gas phase, and 10 per cent sodium hydroxide with a filter paper strip was placed in the center well. No difference could

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be found between oxygen uptake rates with air or 100 per cent oxygen in the
gas phase.

In glycolysis experiments the buffer used for the incubation was a modi-
fied Krebs-Ringer-phosphate-bicarbonate medium of the following compo-
sition: 0.10 m sodium chloride, 1.3 × 10⁻² m potassium chloride, 1.8 × 10⁻³ m
calcium chloride, 6.5 × 10⁻⁴ m magnesium chloride, 0.02 m sodium bicar-
bonate, and sodium phosphate buffer, 5 × 10⁻³ m, pH 7.4. The gas phase
was 95 per cent nitrogen and 5 per cent carbon dioxide. No difference in
the incorporation of leucine into protein was observed between incubations
carried out in the Krebs-Ringer-phosphate buffer medium with air as the
gas phase and in the phosphate-bicarbonate medium with 95 per cent
oxygen and 5 per cent carbon dioxide as the gas phase.

RESULTS AND DISCUSSION

Activation of Amino Acid Incorporation by Anaerobic Glycolysis—The
incorporation of amino acids in vitro into the proteins of a number of tissues
was reported to be inhibited by anaerobiosis (1). In most of these studies
neither was glucose added nor was the rate of glycolysis determined. Le-
Page suggested that anaerobic glycolysis can support the incorporation of
glycine into the protein of several tissues (6). However, half of the activity
of the isolated proteins was lost after performate oxidation. This activity
may have been due in large part to disulfide linkage with radioactive glu-
tathione, for it is known that anaerobic glycolysis can furnish the energy
necessary for glutathione synthesis (7). More recently Zamecnik and
Keller have reported (8) that a rat liver particulate preparation could
support the incorporation of leucine into protein for short periods under
anaerobic conditions if glycolytic intermediates were added to the incuba-
tion medium.

As shown in Table I, anaerobic conditions without added glucose com-
pletely abolished the incorporation of leucine, valine, lysine, phenylalanine,
and methionine into the proteins of the Ehrlich ascites carcinoma. When
sufficient glucose was present, the glycolysis rate was linear for 30 to 40
minutes, but tapered off slightly afterward (Fig. 1). When the glucose
concentration was decreased below that necessary to maintain glycolysis
for 1 hour, the initial glycolysis rate remained constant until an equivalent
amount of acid was produced. In addition, no difference in the incorpora-
tion of leucine was observed when the glucose concentration was increased
6-fold above that necessary for optimal glycolysis. Under conditions of
optimal glucose concentration the incorporation of amino acids varied from
60 to 72 per cent of that supported by oxidation of endogenous substrate.
The specific activity of the protein remained constant upon performate
oxidation.
If the concentration of glucose was decreased to permit glycolysis to continue for only a portion of the incubation period, the incorporation of leucine was lower than would be anticipated from the proportional extent of glycolysis. This is evident from the nature of the induction period observed for incorporation under anaerobic conditions (Fig. 1). When the concentration of glucose was not sufficiently high to maintain glycolysis beyond that required for the induction period (about 15 minutes), the termination of incorporation coincided with that of glycolysis. The cause of the induction period for incorporation is not certain at present, but it is of interest to note that this period did not occur under the usual conditions with oxidation-linked incorporation (see Curve 1, Fig. 3). In addition it is evident from Fig. 1 that the delay in incorporation was not due to an induction period in glycolysis itself. The delay also does not appear to be due to the accumulation of a high energy form of the amino acid. If such were the case, a continuation of incorporation following the end of glycolysis under conditions of low glucose concentration might be anticipated. The induction period appears to be related to the preliminary equilibration period of the cells in the side arm of the Warburg apparatus, where the cells were kept for 10 minutes without substrate. A less marked induction period was also elicited under aerobic conditions by keeping the cells in the side arm for a longer period without adequate oxygenation.

This induction period, which accompanied glycolysis-linked incorporation but not oxidation-linked incorporation under the conditions studied,
leads to lower values for the glycolysis-linked incorporation, as shown in Table I. Independent experiments demonstrated that optimal rates of incorporation of leucine under both oxidative and glycolytic conditions are equal.

**Figure 1.** The effect of glucose concentration upon the rate and duration of anaerobic glycolysis and the rate of incorporation of leucine into protein. Curve 1, anaerobic glycolysis; glucose concentration 30 μmoles in 2 ml. total volume. Curve 2, incorporation coupled to glycolysis of Curve 1. Curve 3, anaerobic glycolysis; glucose concentration 5 μmoles in 2 ml. total volume. Curve 4, incorporation coupled to glycolysis of Curve 3. Incorporation expressed in micromoles of leucine per gm. of protein. Leucine concentration, $1 \times 10^{-3}$ M.

**Nature of DNP Inhibition**—In the experiments discussed in the following section, DNP was employed to dissociate oxidation from phosphorylation, thus causing the incorporative process to function under conditions of a suboptimal energy supply. It was therefore necessary to ascertain whether the concentration of inhibitor used could inhibit the incorporative process directly. The following observations are pertinent to this consideration.

When several concentrations of DNP were used in the medium of a system incorporating leucine into protein, the major extent of the inhibition occurred when the oxygen uptake was stimulated (Fig. 2). Identical results were obtained when valine was the amino acid incorporated. Such
a result would be anticipated if DNP released the oxidation-regulating function of phosphorylation (9, 10). The data resemble those obtained by Frantz and coworkers with rat liver slices (11). The inhibition caused by DNP did not resemble the immediate competitive inhibition caused by amino acid analogues (5), but increased with time (Fig. 3) and was non-

![Diagram](http://www.jbc.org/)

**Fig. 2.** The effect of dinitrophenol on the incorporation of leucine into protein during glycolysis and during aerobic oxidation. Curve 1, anaerobic glycolysis, glucose concentration, 30 μmoles in 2 ml. total volume; 100 per cent = 710 μl. of carbon dioxide evolved. Curve 2, oxidation; 100 per cent = 208 μl. of oxygen uptake. Curve 3, glycolysis-linked incorporation; 100 per cent = 4.2 μmoles incorporated per gm. of protein. Curve 4, oxidation-linked incorporation; 100 per cent = 7.7 μmoles incorporated per gm. of protein. Time of incubation, 1 hour; leucine concentration, $1 \times 10^{-3}$ M.

competitive (Fig. 4). An agent which depleted the energy supply of the cell would be expected to produce a non-competitive, gradually increasing inhibition.

Since the formation of high energy phosphate accompanying anaerobic glycolysis is not inhibited by DNP (12, 13), the effect of this inhibitor upon glycolysis-activated amino acid incorporation might be used as a criterion for a possible direct inhibition of the incorporative process. However, even under anaerobic conditions any adenosinetriphosphate (ATP) formed during glycolysis would be in equilibrium with the oxidative phosphorylation
Fig. 3. The effect of time of incubation upon the inhibition by dinitrophenol of leucine incorporation into protein. Curve 1, incorporation rate of aerobic incubation; leucine concentration, $1 \times 10^{-3}$ M. Curve 2, as above, in the presence of $5 \times 10^{-6}$ M dinitrophenol. Incorporation expressed in micromoles of leucine per gm. of protein.

Fig. 4. The effect of a preincubation with dinitrophenol upon the subsequent incorporation of leucine at several concentrations. Curve 1, in the presence of $5 \times 10^{-6}$ M dinitrophenol. Curve 2, uninhibited oxidation-linked incorporation of leucine. Preincubation with dinitrophenol, 30 minutes; incubation with leucine, 15 minutes. Incorporation expressed in micromoles of leucine incorporated per gm. of protein during the 15 minute incubation period.
mechanism and could therefore be depleted by the DNP-induced hydrolysis of its high energy linkages (14). An inhibition of the glycolysis-linked incorporation of leucine was observed, but its extent in the presence of various concentrations of DNP indicated that it did not directly affect the incorporative process. As the inhibitor concentration was increased, the degree of DNP inhibition of glycolysis-activated leucine incorporation reached a limiting value of 30 per cent, while the inhibition of oxidation-linked incorporation rapidly approached completion (Fig. 2). Such a relationship would be anticipated if the ATP formed during glycolysis could have been dissipated through the DNP-induced breakdown. Under these conditions a limiting rate of high energy phosphate depletion would preclude the complete removal of ATP from the incorporative system. Under oxidative conditions, however, a direct block in the sequence of ATP formation occurred, and none was available to support incorporation at the higher DNP concentrations. No such difference between oxidation and glycolysis-linked incorporation should have been observed if DNP affected the incorporative process directly.

**DNP Inhibition of Multiple Amino Acid Incorporation**—It was previously suggested (5), on the basis of the extent of mutual inhibition, that leucine and phenylalanine could not be activated at the same enzyme site prior to incorporation. The experiments reported here are designed to ascertain whether the same source of energy can be used for preliminary activation of different amino acids. For this the effect of energy depletion by DNP upon the incorporation of amino acids, both singly and in groups, was determined.

When amino acids were present together in the medium at equal concentrations, they were incorporated into Ehrlich ascites protein independently. Similar results were obtained by Borsook and coworkers (15) with bone marrow cells. However, if amino acids were competing for an energy source limited by DNP, groups of amino acids should no longer be activated independently. One would expect that under these conditions the total quantity activated should remain constant. This would occur if the limiting supply of energy must be distributed between the amino acids in this group. Such a redistribution of energy would seriously interfere with the independent incorporation of amino acids.

The extent of interference of incorporation of one amino acid which would be caused by an additional amino acid under limiting energy conditions should depend upon the nature of the incorporative process itself. Two possibilities may be considered. If incorporation is an exchange process between independently activated amino acids and residues in the protein molecule, then, under limiting energy conditions, the total amount of amino acids incorporated when more than one is added to the medium would be the same as when only one is added. Essentially the redistribu-
tion in energy should result in a redistribution of the amino acids being incorporated, but the total number of micromoles of all amino acids being incorporated would not be changed. Incorporation may also be considered to be a synthetic process involving the simultaneous incorporation of endogenous amino acids which are in low concentration. In this case, an amino acid added to the medium in high concentration should effectively compete with the former for the limiting energy and result in a yet more pronounced inhibition of incorporation of groups of added amino acids.

**Table II**

*Effect of Dinitrophenol upon Incorporation into Protein of Amino Acids When Present Singly and in Groups*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Incorporation, μmoles per gm. protein per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In buffer only</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Leucine 10⁻³ M</td>
<td>7.6</td>
</tr>
<tr>
<td>Valine 10⁻⁵ M</td>
<td>5.9</td>
</tr>
<tr>
<td>Leucine 10⁻³ M + valine 10⁻³ M*</td>
<td>14.0</td>
</tr>
<tr>
<td>Leucine 5 × 10⁻³ M</td>
<td>11.2</td>
</tr>
<tr>
<td>Valine 5 × 10⁻³ M</td>
<td>7.4</td>
</tr>
<tr>
<td>Phenylalanine 5 × 10⁻³ M</td>
<td>3.5</td>
</tr>
<tr>
<td>Leucine 5 × 10⁻³ M + valine 5 × 10⁻³ M + phenylalanine 5 × 10⁻³ M†</td>
<td>19.8</td>
</tr>
</tbody>
</table>

* Obtained by summing the values for flasks containing leucine-C¹⁴ + valine-C¹² and leucine-C¹³ + valine-C¹⁴.
† Obtained in the manner indicated above. The incubation period was 1 hour.

These conjectures were not borne out experimentally, for, when more than one amino acid was incorporated into protein in the presence of DNP, the total amount of amino acid incorporated increased and approached or exceeded the values for the uninhibited system when amino acids were incorporated singly. Representative experiments demonstrating this phenomenon are presented in Table II. From the above reasoning and the results reported in Table II it may be inferred that the energy for incorporation is not distributed to the various amino acids under competitive conditions, but in a manner by which it may be allocated to the amino acids which are protein constituents. Such an allocation could occur by activation of sites either on a template (4) or on an enzyme which is specific for incorporation of an amino acid into a peptide. The latter case has been demonstrated by the formation of glutathione from γ-glutamylcysteine and glycine (16). Our observations would appear to preclude preliminary
amino acid activation either as phosphorylated derivatives (2) or by a transpeptidation type mechanism (3).

SUMMARY

The incorporation of radioactive leucine, valine, lysine, phenylalanine, and methionine into the protein of the Ehrlich ascites carcinoma is supported anaerobically under conditions of active glycolysis. An induction period in the glycolysis-linked incorporation is described.

Dinitrophenol inhibits oxidation-linked incorporation almost to completion in concentrations at which it stimulates oxygen uptake. It is a poor inhibitor of glycolysis-linked incorporation. These facts together with others indicate that dinitrophenol does not inhibit the incorporative process directly, but only depletes its required energy supply.

Under conditions of limiting energy supply brought about with dinitrophenol, groups of amino acids are incorporated into protein independently. This is interpreted as an indication that individual amino acids do not compete with each other for activation, but that this activation is allocated to the amino acids which are protein constituents.

BIBLIOGRAPHY

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