The dissociation of respiration from synthetic processes by azide was shown by studies of Clifton in 1937 (1) and by the results of subsequent investigations (2-4). Hotchkiss in 1944 made the significant observation that, although bacterial respiration was enhanced by azide, inorganic orthophosphate (P$_i$) uptake was decreased (5). The ability of azide to uncouple oxidation of substrates from net adenosinetriphosphate (ATP) synthesis in intact cells and cell-free preparations has been well established; present evidence suggests that the uncoupling action of azide, like that of 2,4-dinitrophenol, is on the electron transport between reduced co-enzymes and oxygen (6-9). The uncoupling would result if a phosphorylated intermediate were rendered unstable or if the initial uptake of P$_i$ did not occur although electron transfer continued. Experiments labeled with P$_i$ labeled with P$^{32}$ and O$^{18}$ which favor the latter possibility are presented in this paper.

A second objective was to examine the relation of azide to phosphorylations accompanying anaerobic glycolysis. Data obtained by Spiegelman, Kamen, and Sussman have been interpreted as showing that azide dissociates anaerobic glycolysis from ATP synthesis (3). This suggestion, which has been accepted in more recent publications (10, 11), is considered untenable in view of the experimental results presented herein.

Materials and Methods

Manometric measurements were made with the Warburg apparatus by conventional techniques. P$_i$ was determined essentially as described by Lowry and Lopez (12) or with the use of ferrous sulfate as a reducing agent according to Sumner (13). In experiments with yeast acetone powders chilling of the flasks before addition of an equal volume of 0.6 M trichloroacetic acid was necessary to obtain clear supernatant solutions. The

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295
enzymic reaction was conveniently stopped by addition of sufficient HCl to lower the pH to about 3 before chilling the flasks.

Sodium azide (Eastman) was used without further purification in the experiments with yeast and after crystallization from ethanol-water in experiments with liver mitochondria.

Mitochondria were prepared from fasted rat livers which were minced and ground in 0.25 M sucrose containing $10^{-4}$ M ethylenediaminetetraacetate essentially as described by Schneider (14), except that the initial residue of nuclei and cellular debris was not reextracted. The fluffy layer of microsomes was swirled off and discarded after sedimentation of the mitochondria in a Servall high speed centrifuge and the mitochondria were washed twice by resuspension in the sucrose medium. For the enzyme runs the washed mitochondria were resuspended in a volume (in ml.) of 0.25 M sucrose equal to the gm. of fresh liver. Emphasis in the preparation was on isolation of "clean" mitochondria, not on isolation of high yields.

The yeast acetone powder was prepared by a modification of the procedure of Hochster and Quastel (15). A suspension of fresh brewers' yeast was chilled to 6° and filtered through a Büchner funnel in a cold room, and the yeast was washed three times by suspension in ice-cold 0.85 per cent NaCl, stirred for 5 minutes, and refiltered. 20 gm. portions of the washed yeast were ground vigorously for 10 minutes in a chilled Pyrex mortar with an approximately equal volume of 20 to 30 mesh Pyrex glass and 10 ml. of 0.11 M nicotinamide solution, pH 7.0. The ground yeast was mixed with an equal volume of 0.85 per cent NaCl and the slurry was centrifuged. The milky supernatant liquid was poured into 6 volumes of vigorously stirred ice-cold acetone. The precipitate was collected by centrifugation, washed twice by suspension and centrifugation from cold acetone, transferred to a Büchner funnel with aid of acetone, and washed with cold acetone. The acetone was removed by drawing air through the cake, and the product was dried in a vacuum desiccator over P₂O₅ and stored in a refrigerator. Before use the acetone powder was triturated in a ratio of 25 mg. of powder per 0.3 ml. of water.

For measurement of the amount of P₃₂ present in ATP, the ATP in the trichloroacetic acid supernatant solutions was adsorbed on 100 mg. of charcoal (16), followed by three careful washings to remove Pi. The washed charcoal was transferred to a planchet with the aid of several small volumes of alcohol, and the samples were dried under an infra-red lamp and counted with a Geiger tube by conventional techniques. Azide did not interfere with the adsorption of ATP on the charcoal. Measurements of the amount of O₁₈ in the inorganic phosphate were made by procedures which allowed use of samples of 50 to 100 μmoles of phosphate without further dilution (17); the estimated maximal error of the results given in Table II is about 5 per cent.
Results

Azide and Inorganic Phosphate Requirement of Glycolysis—The suggestion that azide may cause formation of P₁ from 1,3-diphosphoglycerate in the presence of the enzyme 3-phosphoglycerate kinase (3), or formation of P₁ accompanying any of the reactions of glycolysis, may be conveniently checked by measurement of the dependence of glycolysis upon P₁ in the presence of high concentrations of azide. In yeast extracts deficient in phosphatase activity, which metabolize glucose in accordance with the well known Harden-Young equation, 2 glucose + 2 phosphate → 2CO₂ + hexose diphosphate + 2C₅H₁₀OH + 2H₂O, the fermentation rate decreases as the supply of phosphate is exhausted, even though excess glucose remains (18, 19). Any agent that would dissociate glycolysis from P₁ uptake or hasten the liberation of P₁ from organic combination would permit prolonged and enhanced CO₂ production from systems deficient in P₁ or in phosphate acceptors.

The results given in Fig. 1 clearly demonstrate the inability of azide to reduce the P₁ requirement for glucose fermentation by a suitable yeast extract preparation. Even in the presence of 0.025 M azide the rate of CO₂ production fell off as the P₁ supply was exhausted and was stimulated by addition of excess P₁ in a manner entirely comparable to that found in the absence of azide. The results reported in Fig. 1 were obtained with air as a gas phase; entirely similar results were obtained with 95 per cent N₂-5 per cent CO₂ as the gas phase.

Azide and Phosphate Acceptor Requirement for Glycolysis—A further test of the possibility that azide may accelerate the liberation of P₁ from phosphorylated intermediates and the possibility that azide might act as a phosphate acceptor in reactions accompanying glycolysis was made with a reaction system similar to that used for the experiments reported in Fig. 1, except that fructose-1,6-diphosphate was used as a substrate in the presence of excess P₁. Under these conditions the rate of CO₂ production is limited by the action of phosphatases to give phosphate acceptors (19). The data presented in Fig. 2 show that the low rate of CO₂ production was actually slightly decreased by the presence of azide. The dependence of the rate of CO₂ production upon a phosphate acceptor is shown by the marked acceleration of the rate following addition of glucose. Excess fructose-1,6-diphosphate was already present; thus the contribution of glucose was other than as a source of metabolites for CO₂ production.

Effect of Azide on Enzymic Hydrolysis of ATP and Other Phosphates—The rate of hydrolysis of ATP by washed liver mitochondria is greatly accelerated by 2,4-dinitrophenol (8, 20-22). Preliminary experiments showed that azide over a range of concentrations did not cause a hydrolysis of ATP comparable to that produced by 2,4-dinitrophenol but that 3 to
6 \times 10^{-4} \text{ M} \text{ azide did increase ATP hydrolysis by liver mitochondria. This suggested that the higher concentrations of azide might be inhibitory to the reactions causing formation of } P_i \text{ from ATP; subsequent experiments showed that } 5 \times 10^{-3} \text{ M azide blocked the action of 2,4-dinitrophenol. Representative results showing the effects of azide are given in Table I. These data show the marked acceleration of ATP hydrolysis by } 3 \times 10^{-6} \text{ M } 2,4\text{-dinitrophenol, the smaller acceleration by } 5 \times 10^{-4} \text{ M azide, and the small or negligible effect of } 5 \times 10^{-3} \text{ M azide. In addition the data show that } 5 \times 10^{-4} \text{ M azide partially blocked and } 5 \times 10^{-3} \text{ M azide nearly completely blocked the increase in ATP hydrolysis in the presence of dinitrophenol.}

Early in the studies of the action of azide, the possibility that azide might accelerate the action of phosphatases was investigated by measurement of the rate of hydrolysis of various organic phosphates by intestinal
phosphatase. The experiments showed no acceleration of the hydrolysis rate and will not be reported in detail here. Under the conditions used, 0.05 M azide at pH 9.3 had no effect on the rate of hydrolysis of 0.0012 M ATP by intestinal phosphatase and inhibited the hydrolysis of 0.00125

![Graph](image)

**Fig. 2.** Inability of azide to reduce the phosphate acceptor requirement for hexose diphosphate fermentation by yeast extract. To the main compartment of each Warburg flask were added 50 μmoles of fructose-1,6-diphosphate, 1 μmole of ATP, 1 μmole of MgSO₄, 0.5 μmole of diphosphopyridine nucleotide, 10 μmoles of potassium phosphate, and azide as indicated in a total volume of 1.7 ml. at pH 6.5, except for one flask which had a total volume of 1.5 ml. in the main compartment and 0.2 ml. of glucose solution in the center well. Following 15 minutes equilibration at 30°, 0.3 ml. of a suspension containing 25 mg. of an acetone powder of yeast extract was added from the side arm and measurement of CO₂ output commenced 5 minutes later; 55 minutes later glucose was added to one flask from the center well. The gas phase was 95 per cent N₂-5 per cent CO₂.

M fructose-1,6-diphosphate by 21 per cent and the hydrolysis of 0.012 M β-glycerophosphate by 42 per cent.

**Inhibition of P³² and O¹⁸ Exchange Reactions by Azide**—Experiments reported elsewhere have demonstrated that washed liver mitochondria, in the absence of oxygen uptake, catalyze a rapid exchange of P₁ with ATP and of the oxygens of P₁ with water (23). The oxygen exchange reaction has been attributed principally to the reversibility of the primary
EFFECT OF AZIDE ON PHOSPHORYLATION

**Table I**

Effect of Azide and 2,4-Dinitrophenol on Phosphate Cleavage from ATP by Liver Mitochondria

To 0.6 ml. of a solution at pH 7.4 containing 6.5 μmoles of potassium ATP, 7.5 μmoles of MgSO₄, 75 μmoles of KCl, and 1 μmole of potassium phosphate were added 0.2 ml. of washed liver mitochondria in 0.25 M sucrose, 10⁻² M ethylenediaminetetraacetate, and 0.005 M β-glycerophosphate, pH 7.2. The mixture was incubated at 28° for 7 minutes, then water, azide, or dinitrophenol solution was added as indicated to give a final volume of 1.0 ml. After an additional 15 minute incubation, 1.0 ml. of cold 10 per cent trichloroacetic acid was added, and Pᵢ was determined on an aliquot of the supernatant liquid. The values for Pᵢ are averages of duplicate analyses; variation of duplicates was less than 2 per cent.

<table>
<thead>
<tr>
<th>Min. after adding azide or dinitrophenol</th>
<th>Azide</th>
<th>2,4-Dinitrophenol</th>
<th>Pᵢ present</th>
<th>Pᵢ formation caused by azide or dinitrophenol</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>2.18</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>5 × 10⁻⁴</td>
<td>0</td>
<td>2.80</td>
<td>0.62</td>
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<tr>
<td>15</td>
<td>5 × 10⁻³</td>
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<td>2.26</td>
<td>0.08</td>
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<tr>
<td>15</td>
<td>0</td>
<td>3 × 10⁻⁵</td>
<td>6.06</td>
<td>3.88</td>
</tr>
<tr>
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<td>3 × 10⁻⁵</td>
<td>4.24</td>
<td>2.06</td>
</tr>
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<td>15</td>
<td>5 × 10⁻³</td>
<td>3 × 10⁻⁵</td>
<td>2.20</td>
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</tr>
</tbody>
</table>

**Table II**

Effect of Azide on P³² and O¹⁸ Exchange Reactions Catalyzed by Mitochondria

The reaction tubes contained in 3 ml. of total volume, pH 7.4, 19 μmoles of potassium ATP, 22 μmoles of MgSO₄, 150 μmoles of KCl, 0.6 ml. of washed liver mitochondria in 0.25 M sucrose containing 10⁻⁴ M ethylenediaminetetraacetate, and 45 μmoles of potassium phosphate labeled with O¹⁸ and P³². The potassium phosphate was added after a preliminary incubation period of 7 minutes, the samples were incubated for an additional 15 minutes at 25°, and then 1 ml. of 30 per cent trichloroacetic acid was added. P³² present in the ATP and O¹⁸ in inorganic phosphate were determined on appropriate aliquots.

<table>
<thead>
<tr>
<th>Min. after adding P¹³⁸O¹⁸</th>
<th>Azide</th>
<th>Pᵢ present</th>
<th>Radioactivity in ATP</th>
<th>Atom per cent excess O¹⁸ in inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>45.0</td>
<td>32</td>
<td>0.35</td>
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<tr>
<td>15</td>
<td>0</td>
<td>47.4</td>
<td>1717</td>
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<tr>
<td>15</td>
<td>5 × 10⁻⁶</td>
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<tr>
<td>15</td>
<td>5 × 10⁻³</td>
<td>47.0</td>
<td>1568</td>
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<td>15</td>
<td>5 × 10⁻⁴</td>
<td>48.2</td>
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<tr>
<td>15</td>
<td>5 × 10⁻³</td>
<td>45.5</td>
<td>242</td>
<td>0.33</td>
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</table>
reaction or reactions by which energy derived from electron transport is coupled to uptake of P\(_1\). Interference of azide in oxidative phosphorylation would be expected to decrease the P\(_3\)\(^{32}\)-ATP exchange reaction. If azide blocked the initial reaction (or reactions) of P\(_1\) uptake, then the phosphate oxygen exchange should likewise be inhibited.

Two preliminary experiments showed that 0.005 M azide inhibited markedly both the exchange of P\(_3\)\(^{32}\) with ATP and the exchange of phosphate oxygens catalyzed by liver mitochondria. The experiments reported in Table II show the effect of graded levels of azide on the exchange reaction of P\(_3\)\(^{32}\) with the phosphate groups of ATP, and the effect of 0.005 M azide on the exchange of phosphate oxygens in the absence and in the presence of azide. For these experiments P\(_1\) labeled with P\(^{32}\) and O\(^{18}\) was incubated with ATP and washed liver mitochondria under the conditions given in Table II. The results show an increasing inhibition of the P\(_1\)-ATP exchange with increase in azide concentration. The sample incubated in the absence of azide showed the expected marked exchange of P\(_1\)-oxygen; the decrease in O\(^{18}\) content was equivalent to an exchange of 67 microatoms of phosphate oxygen. The P\(_1\) oxygen exchange, as well as the P\(_1\)-ATP exchange, was nearly completely inhibited by 5 \(\times\) 10\(^{-4}\) M azide. It is of interest that the P\(_3\)\(^{32}\)-ATP exchange reaction is still considerable in the presence of a concentration of azide (5 \(\times\) 10\(^{-4}\) M) which caused acceleration of ATP hydrolysis.

**DISCUSSION**

Interpretation of the effects of azide on the P\(_3\)\(^{32}\)-ATP and the P\(_1\)O\(^{18}\) exchanges requires an understanding of the reactions involved. These exchange reactions occur without net oxygen uptake and in the absence of added substrates. Evidence, but not conclusive proof, has been obtained that the exchanges result from a reversal of the reactions by which P\(_1\) uptake is coupled to electron transport, the O\(^{18}\) exchange resulting largely from reversal of the reaction or reactions by which P\(_1\) is incorporated into an intermediate and the P\(^{32}\) exchange from reversal of the over-all reactions by which P\(_1\) is incorporated into ATP (23). If these premises are correct, interference of azide with the P\(_3\)\(^{32}\)-ATP exchange reaction would be an expected result in view of the known uncoupling of oxidative phosphorylation by azide (6–8). Measurement of the P\(_3\)\(^{32}\)-ATP exchange would thus give an evaluation of the effect of azide on oxidative phosphorylation independent of its known inhibition of cytochrome oxidase. Further, if the proposed mechanism of the O\(^{18}\) exchange is valid, the ability of azide to inhibit this O\(^{18}\) exchange means that azide can prevent the initial uptake of P\(_1\) coupled with electron transport. This would occur if, in the presence of azide, electron transport continued without participation of
P₁ in the reaction. A similar suggestion has been made for the action of dinitrophenol (23). The need for P₁ when glutamate is oxidized by kidney homogenates in the presence of azide (6) may result from the requirement of P₁ for the phosphorylation accompanying α-ketoglutarate oxidation at the substrate level; this interpretation is supported by the observations of Judah (7). The hypothesis that the uncoupling agents prevent the initial uptake of P₁ is in contrast to the possibility that they cause release of P₁ from a phosphate-containing intermediate.

The demonstration that azide at appropriate concentrations can accelerate the cleavage of P₁ from ATP by liver mitochondria is of importance in the understanding of the biological effects of azide. The depletion of cellular ATP by azide offers a logical explanation for the observations of various workers showing an increased rate of fermentation and utilization of carbohydrate reserves in the presence of azide (2, 10, 24, 25). Increased substrate utilization in the presence of appropriate concentrations of azide would be anticipated in any system in which the rate of substrate utilization was dependent upon the availability of phosphate acceptors or P₁.

The acceleration of the rate of formation of P₁ from ATP by azide follows logically from the premise that under appropriate conditions P₁ and ATP are in dynamic equilibrium through reversal of the reactions by which ATP formation is coupled to electron transport. For such a dynamic equilibrium to exist, energy derived from the formation of P₁ must be preserved in some manner (for example, by reduction of an electron carrier or formation of an energy-rich intermediate not containing the P₁) in order for the P₁ to be reincorporated into the ATP. In the presence of azide, P₁ formed from ATP would not be taken up again because the azide allows the oxidation to occur without P₁ uptake or in some other manner causes dissipation of the energy derived from the ATP cleavage. Similar considerations apply to the action of 2,4-dinitrophenol. The net breakdown of ATP would thus not result primarily from an increased rate of formation of P₁ but from prevention of the reincorporation of the P₁ formed into ATP.

On the basis of present evidence, the hypothesis that azide in intact cells interferes with phosphorylation accompanying glycolysis is unwarranted. The results given in this paper showing the strict dependence of anaerobic glycolysis by yeast extract on adequate concentrations of inorganic phosphate and phosphate acceptors in the presence of high azide concentrations demonstrate conclusively that azide does not interfere with phosphate esterification or transfer reactions by such extracts. Spiegel-

1 Recent data have shown that the P₁-ATP exchange is more sensitive to the inhibitory effects of 2,4-dinitrophenol and of azide than is the P₁ oxygen exchange. Details of these experiments and their significance will be presented later.
man et al. mention that 5 × 10⁻³ M azide did not inhibit phosphate esterification with a yeast extract preparation (3). None the less, these authors suggested that in intact yeast "azide uncouples anaerobic oxidation from synthesis by means of a replacement reaction between azide and the acyl phosphate of diphosphoglycerate while the latter is combined with phosphoferase." They regarded it likely that excessive amounts of dissolved protein in their cell-free preparations bound azide and thus prevented its inhibitory effect. Binding of azide by proteins is highly untenable as an explanation for the lack of effect of the high concentrations of azide used in the experiments reported herein. If the yeast acetone powder used were all protein with an average molecular weight of 100,000, each protein molecule would need to inactivate nearly 100 azide molecules in the experiment reported in Fig. 2 in which 0.025 M azide was used. Further, the ability of much lower azide concentrations, e.g. 5 × 10⁻⁴ M, to dissociate respiration from phosphorylation in intact cells and in liver mitochondria shows that low concentrations of azide may be effective in the presence of considerable protein. Postulation of a greater sensitivity to azide of glycolytic enzymes in the intact cell than in cell-free extracts is without experimental justification.

Observations cited in support of the contention that azide dissociates anaerobic glycolysis from phosphate uptake (3) are all indirect and may have their explanation in other effects of azide. The depletion of the ATP reservoirs of the intact yeast in the presence of azide would offer a possible explanation for many of the observed effects. In many respects the effect of azide on intact cells is similar to that of 2,4-dinitrophenol; unpublished results from this laboratory and data of other workers (7, 22) have demonstrated that phosphate uptake and transfer reactions associated with glycolysis are not affected by 2,4-dinitrophenol.

The actions of azide and of 2,4-dinitrophenol on oxidative phosphorylation are, however, not entirely analogous. The blocking by azide of the acceleration of P_i formation from ATP in the presence of 2,4-dinitrophenol (Table I) shows clearly that azide has an inhibitory effect not shared by 2,4-dinitrophenol. The rate of ATP breakdown is not decreased by increase in the 2,4-dinitrophenol concentration above that required for a nearly maximal effect (20). The secondary effect of azide is probably responsible for the observation that acceleration of the rate of P_i formation from ATP, although appreciable at 5 × 10⁻⁴ M azide, was small at 5 × 10⁻³ M. Azide will block oxygen uptake (26) and its properties responsible for this blocking may be related to the inhibitory effect on ATP breakdown. In addition, Meyerhof has demonstrated inhibition of an "ATPase" of yeast by azide (19), and, as shown herein, azide will inhibit intestinal phosphatase. Thus, although azide and 2,4-dinitrophenol both have the
ability to uncouple phosphorylation from electron transport, their effects on intact cells may differ; such differences have been reported (4, 27).

The technical assistance of Mrs. Madelyn Ferrigan and Mr. A. Lajeune is gratefully acknowledged.

SUMMARY

Azide at concentrations as high as 0.025 M is without effect on the requirement for phosphate acceptors or inorganic phosphate for glycolysis by a yeast extract. From these results and other considerations the conclusion is reached that present evidence does not warrant postulates that azide interferes with phosphorylations associated with glycolysis in extracts or intact cells.

The reversible exchange of P$^{32}$ of inorganic phosphate with ATP catalyzed by washed liver mitochondria, as well as the more rapid exchange of phosphate oxygen as measured with O$^{18}$, was inhibited by $5 \times 10^{-3}$ M azide. If, as has been suggested, these exchange reactions result primarily from a dynamic reversal of oxidative phosphorylation, then azide prevented the initial uptake of inorganic phosphate coupled with electron transport.

Azide caused an appreciable increase in the rate of net formation of inorganic phosphate from ATP by liver mitochondria which was maximal at about $5 \times 10^{-4}$ M azide but small at $5 \times 10^{-3}$ M azide. The much more marked acceleration of inorganic phosphate accumulation in the presence of $3 \times 10^{-5}$ M 2,4-dinitrophenol was partially blocked by $5 \times 10^{-4}$ M azide and nearly completely blocked by $5 \times 10^{-3}$ M azide. The accumulation of inorganic phosphate may result from a reversal of the phosphate incorporation into the ATP coupled with electron transport and prevention of the uptake of the inorganic phosphate formed. Azide is regarded as having a secondary inhibitory effect, not shown by 2,4-dinitrophenol, on the reactions involved.

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