Orton, 1968), it is evident that the sensitivity of the disulfide bonds joining the active sites to the 5-Ag particle and the weakness of the individual sites have obscured the true nature of the sites themselves and continue to do so. We still have almost no information on their active structures.

We do know that protein is an important part of the structural matrix which holds the active agglutination sites on cells of mating type 5. Proteases can liberate 5-Ag from the cells, and can also reduce the size of the large ($M = 10^8$) agglutinative particles obtained by treating cells with snail enzyme, without a large drop in agglutinative activity. The presence of disulfide bonds on 5-Ag also suggests a protein structure, and the correspondence of 1 bond per 1.7S fragment suggests that this protein may be part of the unique structure at the active site, though not necessarily the active site itself.

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Inhibition of Mitochondrial Energy-Linked Functions by Arsenate. Evidence for a Nonhydrolytic Mode of Inhibitor Action*

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ABSTRACT: Intact rat liver mitochondria and sonicated particles from beef heart mitochondria catalyze a prominent arsenate $\rightleftharpoons$ water exchange in the absence of an energy source. Unlike the phosphate $\rightleftharpoons$ water exchange, the arsenate $\rightleftharpoons$ water exchange reaction does not require ADP. These observations are in line with previous findings on the insensitivity of arsenate $\rightleftharpoons$ water exchange to dinitrophenol and oligomycin and provide no evidence to support the idea that the exchange is a partial reaction of oxidative phosphorylation indicative of arsenate activation to form, for example, a covalent high-energy arsenyl derivative with enzyme or with ADP. Two distinct modes for arsenate inhibition of energy-linked functions were noted. Arsenate inhibition of phosphate-requiring reactions (oxidative phosphorylation and phosphate $\rightleftharpoons$ ATP exchange) was competitive with phosphate as expected.

However arsenate inhibition of energy-linked reduction of NAD^+ by succinate (driven by ATP) was not relieved by phosphate. Phosphate and arsenate exerted identical inhibitory effects on energy-linked reduction and both compounds required the presence of ADP for their inhibitory effects to be displayed. The first mode of inhibition (of phosphate-requiring reactions) does not require the postulation of an arsenolytic reaction and the second mode of inhibition (of energy-linked reduction) is at variance with this type of arsenate induced hydrolytic action.

Arsenate (As_i)[1] has been shown to substitute for phosphate in the stimulation of glycolysis by soluble yeast extracts (Harden and Young, 1906). Arsenate, unlike phosphate, was not incorporated into organic form. It was later suggested that the effect of arsenate on glycolysis was associated with an uncoupling of an oxidative reaction from ATP synthesis at the triose phosphate level (Needham and Pillai, 1937).

This proposal was substantiated by the demonstration that arsenate stimulated the oxidation of glyceraldehyde 3-phosphate in the absence of phosphate (Warburg and Christiansen, 1939). These findings led to the concept of "arsenolysis" to describe the hydrolytic breakdown of an unstable arsenyl analog of a naturally occurring phosphoryl compound (Doudoroff *et al.*, 1947). Early studies of the effect of arsenate on mitochondrial systems showed that relatively high concentrations of arsenate stimulated respiration and decreased phosphorylation (Crane and Lipmann, 1953). A stimulation by arsenate of mitochondrial ATPase was investigated using arsenate labeled with ^{18}O (Itada and Cohn, 1963). As a control,

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: As_i, inorganic arsenate; DNP, 2,4-dinitrophenol.

TABLE I: Lack of ADP Requirement for the Apparent $As_i \rightleftarrows H_2O$ Oxygen Exchange Catalyzed by Submitochondrial Particles.

App Exchange	Reaction Mix.	% Isotopic Equil
$P_i \rightleftarrows [^{18}O]H_2O$	Complete	74
$P_i \rightleftarrows [^{18}O]H_2O$	ADP omitted	2.4
$H_2O \rightleftarrows [^{18}O]As_i$	Complete	31[a]
$H_2O \rightleftarrows [^{18}O]As_i$	ADP omitted	34[a]

[a] These values are not corrected for the zero time value, which in this experiment was 11% of isotopic equilibrium. Incubations were carried out at 30°, pH 7.5 for 5 min in medium containing 0.25 M sucrose, 19 mM Tris-sulfate, 5 mM $MgSO_4$, 10 mM succinate, and 2–3 mg of washed particles from sonicated beef heart mitochondria. ADP (1 mM) was added where indicated. Final volume was 1 ml. Incubations were carried out in the presence of either 10 mM P_i + 0.84 atom % excess ^{18}O in H_2O, or 30 mM As_i (approximately 60 atom % excess ^{18}O). Additions were made as described in Table II except that a "zero time" value for $H_2O \rightleftarrows [^{18}O]As_i$ exchange was measured on samples removed within 5 sec following addition of particles.

the effect of arsenate on the coupled system glyceraldehyde 3-phosphate dehydrogenase–1,3-diphosphoglycerate kinase was tested. In the control experiment appearance of ^{18}O label in P_i correlated well with As_i-induced ATPase. Results with mitochondrial ATPase were inconclusive due to presence of mitochondrial-catalyzed $As_i \rightleftarrows H_2O$ exchange. Unlike the $P_i \rightleftarrows H_2O$ exchange, the $As_i \rightleftarrows H_2O$ exchange is not inhibited by oligomycin or by DNP (DeMaster and Mitchell, 1970). Moreover, other workers have concluded that the observed stimulation of mitochondrial ATPase is largely an artifact and it has been concluded from this and other findings that arsenate studies provide the best evidence to date for the participation of a phosphorylated intermediate (Ter Welle and Slater, 1964). This conclusion has been reaffirmed in later studies (Cross and Wang, 1970) and similar work has also served to support the postulate that at least two nonphosphorylated intermediates are involved in ATP synthesis (Ter Welle and Slater, 1967). This paper reports studies directed at finding evidence for nonhydrolytic modes of As_i action.

Experimental Section

Sonicated particles were prepared from heavy beef heart mitochondria as was described previously (Smith and Hansen, 1962). Rat liver mitochondria were isolated in 0.25 M sucrose containing 0.1 mM EDTA (pH 7.5) and were washed three times with 0.25 M sucrose before use. Protein was determined by the method of Lowry *et al.* (1951).

Oxygen-uptake studies were done using a Gilson Medical Electronics Oxygraph equipped with a Yellow Springs Instrument Co. bath assembly and electrode.

Highly enriched $[^{18}O]KH_2AsO_4$ was prepared by equilibration of the salt with $[^{18}O]H_2O$. Nonenzymatic exchange was minimized during manipulation by dissolving the salt in cold KOH before use and by adding sufficient H_2SO_4 to the incubation mixture to bring the final pH to 7.5. Arsenate $\rightleftarrows$ water oxygen exchange was measured by removing 0.1-ml aliquots at various times and freezing the samples in liquid nitrogen. Water was subsequently distilled off at −15° under reduced pressure. The ^{18}O content of water and of phosphate was measured using guanidine hydrochloride (Boyer *et al.*, 1961). The $P_i \rightleftarrows$ ATP exchange was measured by the method of Sugino and Miyoshi (1964). Excess ammonium molybdate and triethylamine were used to insure complete precipitation of phosphate in the presence of arsenate.

Nucleotides were obtained from commercial sources and their purity was checked by appropriate coupled enzyme assay. Estimation of ATPase in intact mitochondria was done in presence of KCN (in presence or absence of arsenate) using pyruvate kinase, adenylate kinase, and lactate dehydrogenase to measure the amount of ADP + AMP formed from ATP. NADH disappearance was measured using a Cary Model 15 spectrophotometer with the 0–0.1 slide-wire. Estimation of ATPase in submitochondrial particles was performed by measuring P_i released using an isobutyl alcohol–benzene-extraction procedure (Lindberg and Ernster, 1956). Arsenate interference with the colorimetric analysis of P_i at 310 mμ was minimized by carrying out the P_i extraction with isobutyl alcohol–benzene at 0° and by quickly removing the organic from the aqueous layer. Where necessary, P_i for ^{18}O analysis was separated from the bulk of As_i present by repeated selective precipitation of P_i as the triethylamine salt of the phosphomolybdate complex. The P_i salt was precipitated at room temperature, whereas the As_i salt was precipitated only on heating. Traces of As_i that had coprecipitated with the P_i salt were removed by converting the phosphomolybdate complex into the magnesium ammonium salt and treating the latter with Zn–HCl until AsH_3 evolution ceased. The P_i was then reprecipitated as the triethylamine phosphomolybdate complex and this was purified as previously described (Mitchell *et al.*, 1967).

Results

In independent studies Mitchell *et al.* (1967) and Hinkle *et al.* (1967) noted a nearly complete nucleotide requirement for respiration-supported $P_i \rightleftarrows H_2O$ exchange in sonically prepared submitochondrial particles. It has been proposed that this exchange represents at least in part a manifestation of phosphate activation (Mitchell *et al.*, 1967). A lack of a similar nucleotide requirement for particle-catalyzed $As_i \rightleftarrows H_2O$ exchange is shown in Table I. The control experiment demonstrated the capacity of the particles to catalyze an ADP-stimulated $P_i \rightleftarrows H_2O$ exchange. Present evidence suggests that ADP acts as a substrate for the $P_i \rightleftarrows H_2O$ exchange reaction, rather than as an activator (Jones and Boyer, 1969). Apparently ADP plays no similar role in mitochondrial $As_i \rightleftarrows H_2O$ exchange.

A further discrepancy between the arsenate- and phosphate-exchange reactions was found when intact rat liver mitochondria were used in place of beef heart submitochondrial particles. In this experiment (Table II) mitochondria were depleted of high-energy intermediates and endogenous ATP by incubation with KCN in absence of added ATP. Absence of added ATP as an energy source is important since anaerobiosis produced by KCN is insufficient to block mitochondrial $P_i \rightleftarrows H_2O$ exchange (Boyer *et al.*, 1966). The experiment shown in Table II thus reveals no energy requirement for mitochondrial-catalyzed $As_i \rightleftarrows H_2O$ exchange.

The inhibitory effect of As_i on oxidative phosphorylation was reinvestigated to establish whether the reduction in the

TABLE II: Failure of KCN to Inhibit the Apparent $As_i \rightleftarrows H_2O$ Oxygen Exchange in Contrast to the Inhibitory Effect of KCN on the Apparent $P_i \rightleftarrows H_2O$ Oxygen Exchange Catalyzed by Intact Rat Liver Mitochondria.[a]

Reaction Mixture	% Isotopic Equilibrium: At 30 sec	At 5 min, 30 sec
Expt 1: $[^{18}O]As_i \rightleftarrows H_2O$		
1. Complete	27	75
2. Complete + KCN	34	72
3. Mitochondria omitted + KCN	0	0
	At zero time	At 5 min
Expt 2: $P_i \rightleftarrows [^{18}O]H_2O$		
1. Complete	0	43.2
2. Complete + KCN	0	3.7

[a] Incubations were carried out with vigorous shaking at 30°, pH 7.5, in a medium containing 80 mM sucrose, 8 mM Tris-sulfate, 5 mM 3-hydroxybutyrate, 2–3 mg/ml of mitochondrial protein, and 5 mM KCN where indicated. Incubations with P_i additionally contained in a final volume of 2.5 ml, 10 mM P_i and 0.84 atom % excess ^{18}O in medium H_2O. Reactions with P_i were started by addition of mitochondria and were stopped by addition of $HClO_4$ to final concentration of 0.53 N. P_i was isolated and analyzed as described in Methods. Incubations with As_i additionally contained 10 mM $[^{18}O]As_i$ (approximately 60 atom % excess ^{18}O) in a final volume of 1 ml. Reactions were started by addition of mitochondria (zero time) 30 sec after addition of labeled As_i to medium. Samples of H_2O taken at the times specified were analyzed for ^{18}O content as described in Methods.

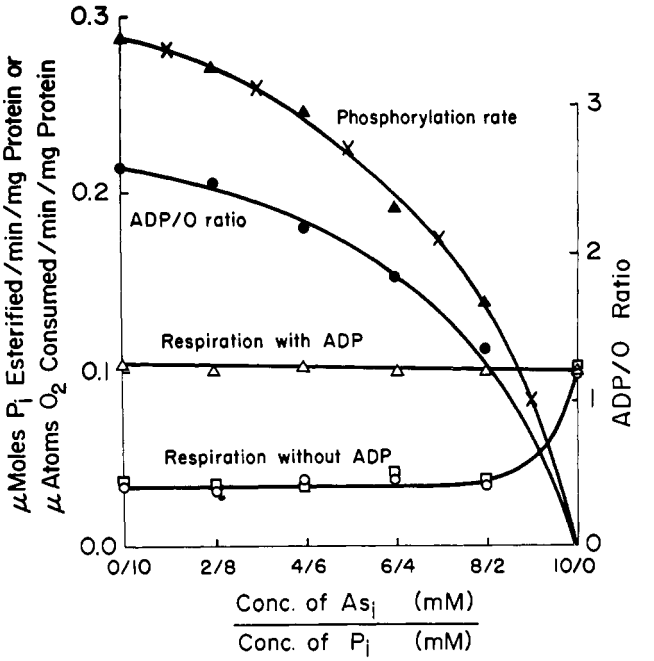

FIGURE 1: Effect of arsenate on ADP:O ratio, phosphorylation rate, and respiration rate of intact rat liver mitochondria. Rat liver mitochondria (2.6 mg) were incubated at 38°, pH 7.5, in a final volume of 2.5 ml of mix containing 0.25 M sucrose, 5 mM 3-hydroxybutyrate, 5 mM $MgSO_4$, 1 mM EDTA, and As_i + P_i to total final concentration of 10 mM as shown. ADP was added in 0.5-μmole amounts in small volumes using a Hamilton microsyringe. (●) ADP:O ratio (▲) rate of phosphorylation; (△) rate of respiration in presence of ADP; (○) rate of respiration before addition of ADP; (□) rate of respiration after esterification of added ADP; (×) rate of phosphorylation calculated on the assumption that As_i (K_i = 3.0 mM) competes with P_i (K_m = 0.7 mM).

ADP:O ratio in the presence of As_i was due to reduction in the rate of phosphorylation, elevation of respiration, or to a combination of both these effects. Results are shown in Figure 1. Half-maximum ADP:O ratio was obtained with a ratio of As_i:P_i = 4:1. Ter Welle and Slater (1967) have reported ratios of 5:1 to 10:1 for a similar effect. Data in Figure 1 show that although the familiar elevation of respiration by As_i is observed in the absence of any added P_i, such an elevation of respiration does not account for the decrease in ADP:O ratio by As_i. The inhibitory action of As_i can be accounted for quite simply on the basis of competitive inhibition with P_i in the phosphorylation reaction. States 3 and 4 respiration rates were essentially unchanged by replacement of up to 80% of P_i by As_i. In this experiment little or no additional respiration was obtained upon addition of ADP to mitochondria in 10 mM As_i although in some other experiments ADP gave about 50% stimulation of respiration as reported by Ter Welle and Slater (1967). Possibly this variability is due to the presence of small amounts of ADP in the incubation mixtures, since the K_m for ADP is quite small (Chance and Williams, 1955).

To pinpoint more precisely the effect of As_i on phosphorylation, a more detailed study was carried out on the effect of As_i on a partial reaction of oxidative phosphorylation, the $P_i \rightleftarrows$ ATP exchange. An ATP-regenerating system was present to prevent accumulation of ADP resulting from ATP hydrolysis. In agreement with the findings of Groot (1969), ADP was found to inhibit $P_i \rightleftarrows$ ATP exchange catalyzed by intact rat liver mitochondria. Inhibition by As_i was found to be competitive with P_i (Figure 2). The exchange reaction appeared to be slightly more sensitive to As_i than was the overall coupled reaction (K_m for P_i = 2.2 mM, K_i for As_i = 1.2 mM, for the exchange reaction, compared to estimated values of K_m = 0.7 mM, K_i = 3.0 mM for the overall reaction).

The inhibitory effect of As_i on ATP-driven reduction of NAD^+ by succinate catalyzed by submitochondrial particles from beef heart is shown in Figure 3. However P_i was equally inhibitory and a mixture of 10 mM P_i + 10 mM As_i was indistinguishable from 20 mM P_i or 20 mM As_i. The marked curvature of the reaction progress curves noted in this figure appears to be due to accumulation of ADP. Presence of a pyruvate kinase ATP-regenerating system prevented inhibition of energy-linked reduction by As_i or P_i. Moreover, addition of phosphoenolpyruvate and pyruvate kinase to the reaction mixture 5 min after the reaction had been started by the addition of ATP resulted in an immediate restoration of the reaction to the initial rate. These experiments were carried out under conditions similar to those described for Figure 3 except that a range of Mg^{2+} concentrations (4–25 mM) was tested. A detailed study of the inhibition of energy-linked reduction by As_i (or P_i) and ADP is in progress.

Several experiments were performed to test the effect of As_i on intact rat liver mitochondrial ATPase in the presence and absence of P_i. Rates of ATP hydrolysis of 0.045–0.05

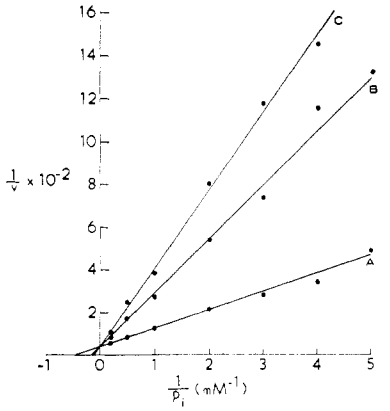

FIGURE 2: Competitive inhibition by As_i of $P_i \rightleftharpoons ATP$ exchange catalyzed by intact rat liver mitochondria. Rat liver mitochondria (3.4 mg) were incubated for 5 min at 38°, pH 7.5, in a final volume of 1.0 ml containing 0.25 M sucrose, 50 mM Tris-chloride, 4 mM ATP, 2 mM phosphoenolpyruvate, 17 μg of pyruvate kinase, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM KCN, 1.6×10^6 cpm of $^{32}P_i$, phosphate as shown in figure, and arsenate as indicated below. The concentrations of arsenate were (A) no As_i, (B) 2.0 mM As_i, and (C) 4.0 mM As_i. Rate of exchange (v) is expressed as μmoles of P_i exchange per min per mg of protein.

μmole of ATP hydrolyzed per min per mg of protein were observed with no discernible trend for the activity to increase upon replacing as much as 90% of a 10 mM P_i buffer with As_i. The potassium salts were used and the incubations were made at 38°. Ter Welle and Slater (1967) reported values of 0.02 μmole/min per mg of protein at 25°. An elevation (70%) of ATPase by As_i in the absence of added P_i has been noted in the present work. Ter Welle and Slater (1967) reported that 5 mM As_i caused elevations of 27–80% above the basal rate of ATP hydrolysis.

Table III shows the inhibition by As_i of the $P_i \rightleftharpoons H_2O$ and $ATP \rightleftharpoons H_2O$ reactions catalyzed by sonicated beef heart mitochondrial particles. In the presence of As_i the final medium P_i concentration was approximately 1.0 mM. This level of P_i should be sufficient to sustain a measurable $P_i \rightleftharpoons H_2O$ exchange, since in other experiments an average level of 0.2 mM

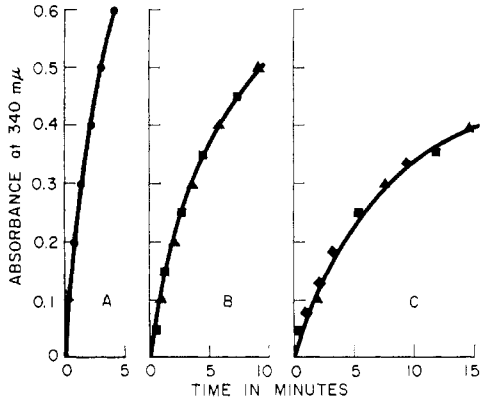

FIGURE 3: Inhibition of ATP-driven energy-linked reduction of NAD^+ by either arsenate or phosphate. Washed submitochondrial particles (1.5 mg) from beef heart were incubated at 25° in a final volume of 2.5 ml of medium containing 0.25 M sucrose, 50 mM Tris-sulfate, 10 mM succinate, 4 mM $MgSO_4$, 4 mM ATP, 1 mM KCN, 1 mM NAD^+, and P_i or As_i as shown. A, no further additions; B, 10 mM P_i or 10 mM As_i; C, 20 mM P_i or 20 mM As_i or 10 mM P_i plus 10 mM As_i. (●) No additions, (▲) P_i, (■) As_i, and (◆) P_i + As_i.

TABLE III: Inhibition by Arsenate of $P_i \rightleftharpoons H_2O$ and $ATP \rightleftharpoons H_2O$ Oxygen Exchanges Catalyzed by Submitochondrial Particles.[a]

Additions (mM)	μmoles of P_i Released	Atom % Oxygen	
		Medium P_i	Acid-Labile Nucleotide P
P_i (10)	6.1	0.099 (0.0153)	0.79
As_i (25)	5.3	0.147 (0.142)	0.007
DNP (1)	8.7	0.173 (0.142)	0.000

[a] Washed particles (5–7 mg) were incubated in a final volume of 5 ml for 10 min at pH 7.5 and at 30° in a mixture containing 0.25 M sucrose, 50 mM Tris-sulfate, 5 mM $MgSO_4$, 4 mM ATP, and other additions as shown. Medium water contained 0.568 atom % excess ^{18}O. Reaction was stopped by addition of 0.5 ml of 5.8 N $HClO_4$. Values shown in parenthesis are those expected for simple hydrolysis with the incorporation of 1 atom of oxygen from H_2O into each molecule of P_i released. Difference between values in parentheses and those without represent oxygen incorporated by exchange.

medium P_i sustained an exchange which incorporated 30% more ^{18}O into P_i than that calculated on the basis of simple hydrolysis (Mitchell *et al.*, 1967). This conclusion was verified in a separate experiment carried out under conditions similar to those described in Table III, in which particles were incubated in the presence and absence of 10^{-4} M *p*-mercuribenzoate. The initial P_i concentration was 2×10^{-6} M and the final P_i concentrations were 1.03 mM (mercurial absent) and 1.68 mM (mercurial present). The ^{18}O contents of the isolated medium P_i samples were in excess of the values calculated on the basis of simple hydrolysis by 84 and 52% (absence and presence of mercurial, respectively). These data lend credence to the view that the inhibitions of the exchange reactions noted in Table III arose from the inhibitory effect of As_i and DNP in the incubation medium rather than from limiting amounts of medium P_i.

Discussion

The idea that As_i inhibits oxidative phosphorylation (and presumably related energy-linked reactions) by a process analogous to the arsenolytic mode of action As_i exerts in substrate level phosphorylation and in other P_i-requiring reactions is well established. In the absence of a well-characterized isolatable arsenic-containing compound and of direct studies on the lability of this compound, evidence for participation of As_i in a hydrolytic reaction is largely indirect and is based on observations of As_i-induced hydrolysis of an appropriate compound (Doudoroff *et al.*, 1947) or of expected oxygen-transfer reactions using ^{18}O-labeled As_i (Slocum and Varner, 1960). However, the marginal effect of As_i on mitochondrial ATPase (compared to the effect of DNP) noted here and by others (Ter Welle and Slater, 1964, 1967) provides no substantial evidence for the presence of an arsenolytic reaction proceeding by the hydrolysis of an unstable arsenyl intermediate.

The presence of a mitochondrial-catalyzed $As_i \rightleftharpoons H_2O$ exchange has been generally regarded as indicative of a partial reaction of oxidative phosphorylation involving As_i activation. Loss of oxygen from As_i to water could occur in at least two ways: loss due to a constant recycling of As_i in an energy-depleting reaction requiring formation and hydrolysis of a high-energy arsenyl compound or loss due to a partial reaction involving the formation of a relatively stable high-energy arsenyl compound. Only in the former case would As_i activation be expected to give rise to As_i-induced ATPase.

The present experiments confirm the original observations of Itada and Cohn (1963) that intact rat liver mitochondria catalyze a prominent $As_i \rightleftharpoons H_2O$ exchange. Phosphorylating particles from sonicated beef heart mitochondria gave a similar exchange (Table I). However, neither in intact mitochondria nor in submitochondrial particles did the reaction appear to be associated with oxidative phosphorylation, as judged by the lack of an ADP or an energy requirement for the exchange. This conclusion is in agreement with the previously reported findings on the insensitivity of $As_i \rightleftharpoons H_2O$ exchange catalyzed by intact rat liver mitochondria to oligomycin and to DNP (DeMaster and Mitchell, 1970). An important piece of evidence for the participation of As_i in an energy-linked activation reaction is thus missing. It is difficult, for example, to reconcile suggestions for the existence of an arsenyl analog of ATP (Lardy *et al.*, 1964) with the data presented in Table I.

Ernster *et al.* (1967) have investigated the effect of As_i on ADP:O ratios in intact rat liver mitochondria. Interpretation of their data is difficult since the double-reciprocal plots employed by these authors are not in fact conventional Michaelis–Menten plots since no term with the dimension of $(\text{velocity})^{-1}$ appears. Ernster *et al.* viewed As_i as an alternative substrate rather than as a competitive inhibitor of P_i. Kinetic constants for As_i (K_m = 2.2 mM) and P_i (K_i = 1.2 mM) were reported. In an attempt to explain the discrepancy between a high rate of $As_i \rightleftharpoons H_2O$ exchange and a low rate of As_i-induced ATP hydrolysis, Ernster *et al.* favored a scheme involving a stable arsenyl intermediate. However, as noted in the present paper, simple competition between P_i and As_i is sufficient to account for the decrease in ADP:O ratio by As_i and the $As_i \rightleftharpoons H_2O$ exchange does not appear to reflect a partial reaction of oxidative phosphorylation. There is thus no compelling reason to postulate the existence of either a stable or an unstable As_i intermediate on this evidence.

Chan *et al.* (1960) have noted approximately 80% inhibition of $P_i \rightleftharpoons H_2O$ exchange by As_i using digitonin particles as the enzyme source. The greater inhibition (about 100%) noted in the present paper doubtless reflects the higher As_i:P_i ratio used in the present work, in contrast to the equimolar amounts of As_i and P_i used by Chan *et al.* It is of interest to note that the oxygen-exchange reactions appear to be more sensitive to As_i than does the ATP-driven energy-linked reduction. In contrast, the $P_i \rightleftharpoons H_2O$ exchange associated with myosin ATPase was not inhibited by As_i (R. A. Mitchell and S. E. Margitic, unpublished data). Studies to determine whether or not oxygen-exchange reactions are mandatory features of ATP-driven energy-linked reduction are in progress.

The small decrease in ATPase in presence of As_i (Table III) has been noted consistently and will be discussed in more detail elsewhere. It should be stressed that this decrease in ATPase was observed using sonically prepared particles. These particles are morphologically and biochemically simpler than intact mitochondria or digitonin particles which possess an As_i-induced ATPase. Possibly some of the effects of As_i on these more complex systems reflect other energy-linked events such as ion movement, as suggested by Sandoval *et al.* (1970) and Kagawa and Kagawa (1969). Possibly As_i (or P_i) acts in conjunction with ADP at a respiration control site.

The finding that inhibition by As_i of ATP-driven energy-linked reduction of NAD^+ by succinate was not reversed by P_i (which appeared to be equally inhibitory) is incompatible with an arsenolytic type of inhibition and points to a second mode of As_i inhibition distinct from the inhibition of P_i-requiring reactions. The possibility that As_i and P_i exert their effects by modifying the kinetic parameters of the energy-coupled system is presently being studied.

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Effects of Macrolides on Peptide-Bond Formation and Translocation*

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ABSTRACT: The formation of *N*-formylmethionylpuromycin and the synthesis of dilysine in the absence of translocation were inhibited by niddamycin, carbomycin, spiramycin, and tylosin, the former two being more potent than the latter two. Erythromycin and oleandomycin had no significant effect on these reactions. In the complete reaction mixture in which oligolysine was synthesized, niddamycin and carbomycin inhibited dilysine synthesis, spiramycin and tylosin caused the accumulation of dilysine but inhibited trilysine synthesis, and erythromycin and oleandomycin caused the accumulation of di-, tri-, and even tetralysine on some occasions, but inhibited the synthesis of longer peptides. Similar results were obtained with polyphenylalanine synthesis. The G- and T-dependent hydrolysis of GTP and enzymic binding of aminoacyl-tRNA were not inhibited by the macrolides. A strict interpretation of these data is that carbomycin and niddamycin inhibited peptide-bond formation, spiramycin and tylosin inhibited peptide-bond formation as well as translocation, and that erythromycin and oleandomycin neither inhibited peptide-bond formation nor translocation but inhibited some other unknown step. This interpretation involving diversified actions of antibiotics in a single family is rather unattractive. Since their structures are similar and they compete for the same binding site on the 50S ribosomal subunit, an alternative hypothesis is proposed. All macrolides inhibit peptide-bond synthesis.

However, the formation of earlier peptide bonds is less sensitive to the macrolides than later peptide bonds, and thus, accumulation of short peptides is caused by less potent macrolides.

The macrolide antibiotics have been shown to inhibit protein synthesis in intact cells and in cell-free systems of bacteria (Brock and Brock, 1959; Taubman *et al.*, 1964; Vazquez, 1966; Mao and Wiegand, 1968; Ahmed, 1968). The specific step affected by the macrolides has not been identified, although the general consensus is that they inhibit some step after the binding of aminoacyl-tRNA to the ribosomes. Two mechanisms of inhibition have been proposed. The first one is based on the inhibition of the formation of poly(Lys)-puromycin (Čërnà *et al.*, 1969), on the addition of a single lysine unit to the ribosomal-bound poly(Lys)-tRNA (Jayaraman and Goldberg, 1968), and on the inhibition of the release of peptides of CpA-Gly[1] (Rychlík *et al.*, 1967). These data implied that the macrolides inhibit the peptide-bond formation step. The second mechanism is based on the lack of inhibition of the puromycin-induced release of nascent peptides in the presence of chlorotetracycline (Cundliffe and McQuillen, 1967), on the inhibition of Ac-triPhe but not Ac-diPhe synthesis (Corcoran and Oleinick, 1969), and on inhibition of tRNA release (Igarashi *et al.*, 1969). These data suggested that erythromycin inhibits translocation. However, D. Schlessinger (1969, personal communication) observed that ribosomes could move on mRNA in erythromycin-treated *Escherichia coli* cells even though peptide synthesis had ceased. A further complication arises by noting that although the macrolides are structurally similar, their action on protein synthesis seems to be different. The formation of F-Met-puromycin from F-Met-hexanucleotide, puromycin, and 50S ribosomes, a stringent test for peptide-bond formation, was inhibited by carbomycin and spiramycin, but not by erythromycin and oleandomycin (Monro and Vazquez, 1967).

It has been proposed that since dipeptide synthesis is a function of peptidyl transferase activity and the synthesis of longer peptides involves translocation, the ability of an antibiotic to inhibit peptide-bond formation or translocation can be determined by quantitative analysis of dipeptide and tripeptide synthesis. Based on this hypothesis, the mechanisms of action of several antibiotics were studied by Pestka (1970b).

In this communication, synthesis of dilysine in the absence of G factor and GTP, or in the presence of fusidic acid, and the F-Met-puromycin reaction were used as tests for the peptide-bond formation step, and synthesis of tri- and longer peptides

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[1] Abbreviations used are: Lys_2, dilysine; Lys_3, trilysine, etc. Lys-tRNA, lysyl-tRNA; poly(Lys)-tRNA, poly(lysyl-tRNA); Phe-tRNA, phenylalanyl-tRNA; Ac-Phe-tRNA, *N*-acetylphenylalanyl-tRNA; Ac-diPhe-tRNA, *N*-acetylphenylalanylphenylalanyl-tRNA; F-Met-tRNA, *N*-formylmethionyl-tRNA; CpA-Gly, cytidylyl-(3′→5′)-2′(3′)-*O*-glycyladenosine; GMPPCP, 5′-guanylylmethylenediphosphonate; 1 optical density unit is the amount of material which in 1.0 ml would yield a value of 1.0 for the optical density measured at 260 mμ in a cuvet with a path length of 1.0 cm.