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2,4-Dinitrophenol and Azide as Inhibitors of Protein and Ribonucleic Acid Synthesis in Anaerobic Yeast*

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ABSTRACT: When *Saccharomyces cerevisiae* was grown under anaerobic conditions with energy and carbon supplied by glucose, it was established that two classic uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and azide, are potent inhibitors of protein and ribonucleic acid (RNA) synthesis. During the inhibition it was found that the cells maintained high levels of adenosine triphosphate (ATP), amino acids, and nucleotides. All of the reactions of *in vitro* amino acid incorporation and stimulation of incorporation by addition of polyuridylic acid (poly U) were insensitive to the uncouplers. The incorporation of added uracil into uridine triphosphate (UTP) and the activity of an isolated deoxyribonucleic acid dependent RNA polymerase were unaffected by the poisons. A uniform depression of [^{14}C]uracil and [^{14}C]leucine incorporation throughout the polysomes and monosomes was revealed by sucrose density gradient studies. Several possible explanations are considered including the idea that high-energy intermediates of oxidative phosphorylation common to ATP production and macromolecular synthesis may exist.

In facultative anaerobes, energy for anabolic processes can be produced either aerobically through the coupled processes of electron transport and oxidative phosphorylation or anaerobically by a fermentation process involving substrate phosphorylation. Both processes culminate in the formation of ATP.[1] Endergonic processes which directly employ ATP should then proceed irrespective of its route of formation.

In this paper we will show that despite high levels of ATP present in yeast under conditions of anaerobic fermentation of glucose, two classic uncouplers of oxidative phosphorylation (in concentrations where they normally would uncouple oxidative phosphorylation in aerobic cells), are capable of preventing protein and RNA synthesis. Previous workers have reported that 2,4-dinitrophenol (2,4-DNP) and azide will inhibit growth and induced enzyme synthesis in yeast under anaerobic conditions (Kovac and Istenesova, 1964; Spiegelman, 1947; Reiner and Spiegelman, 1947). These observations have been confirmed and extended. We have tested the effects of these agents on the maintenance of amino acid and nucleotide pools formed from glucose, the concentration of ATP, all *in vitro* reactions of protein synthesis, with and without stimulation by mRNA, the activity of yeast DNA-dependent RNA polymerase, and the distribution of radioactivity from uracil or leucine in the cytoplasmic supernatant fluid of cells after incubation, by analysis in a sucrose density gradient.

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TCA, trichloroacetic acid; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; AMP, adenosine monophosphate; PPO, 2,5-diphenyloxazole.

Although none of the isolated systems was affected by these poisons, protein and RNA synthesis in the intact yeast were very susceptible to inhibition. The data are suggestive that *in vivo* protein and RNA synthesis is energized *via* a high-energy intermediate(s) similar to or identical with those of oxidative phosphorylation and not by ATP directly. A preliminary report of these studies has been published (Jarett and Hendler, 1965).

Methods

Description of Organism and Growth Conditions. The organism studied was *Saccharomyces cerevisiae* ATCC no. 7754, Baker's yeast. The strain was stored on agar slants of Sabouraud's medium and grown in the 5% glucose medium described by Kovac and Istenesova (1964) minus ergosterol and Tween 80. A starter culture was grown anaerobically overnight by inoculating 6 ml of medium in 15 × 125 mm test tube which contained a magnetic stirring bar. The tube was sealed with a cotton plug which was partially wetted with 0.2 ml of 50% sodium hydroxide to control the pressure due to evolved CO_2. Another portion of the plug was wetted with 7 drops each of 10% sodium carbonate and 50% pyrogallic acid, and finally, the tube was sealed with a rubber stopper to ensure anaerobiosis. The tube could be directly assayed in a Klett–Summerson photometer without disturbing the seal. In the initial experiments, 1 drop of 0.01% methylene blue was added to the tube contents to assure that anaerobic conditions were achieved and maintained. In those cases, the solution was completely decolorized 7–10 min after sealing. All incubations were carried out at 30°. The fully grown culture reached a reading of 400 in a Klett–Summerson photometer with a no. 54 filter. Experiments were performed with an inoculum of 0.5 ml of such a culture in 6 ml of fresh medium in tubes set up as described above.

ATP Assay. Yeast culture (1 ml) was added to 1 ml of boiling 0.02 M glycine buffer (pH 8.4) and boiling was continued for 15 min (Bergmeyer, 1963). The assay solution contained 0.7 ml of boiled extract, 0.1 ml of Sigma firefly lantern extract (FLE), and 0.2 ml of a solution consisting of one part of 0.2 M glycine buffer (pH 7.4), five parts of 0.1 M $NaHAsO_4$ (pH 7.4), and four parts of 0.05 M magnesium sulfate. The intensity of emitted light as measured in an Aminco-Bowman spectrophotofluorometer, Model no. 4-8100, decayed only to the extent of about 5% over a 45-sec period. The intensity at 15 sec was used as the end point in our assay. A linear relation for ATP was found in the range from 1 to 32 mμmoles. ADP had virtually no effect in this range and ATP added to the yeast suspension was quantitatively recovered even in the presence of dinitrophenol and azide. It was found that storing the suspension on ice, prior to extraction, resulted in a loss of ATP and so the suspension was always extracted immediately.

Preparation and Incubation of the Ribosomal Amino Acid Incorporating System. Culture (44 l.) was grown aerobically, harvested, and the cells were subsequently stored at −80°. The preparation and requirements for optimal incubation conditions for an acellular amino acid incorporating system from yeast have been described elsewhere in detail (Lucas *et al.*, 1964; Dietz *et al.*, 1967). The composition of the incubation mixture consisted of the following in micromoles per milliliter: creatine phosphate, 5; creatine kinase, 50 μg; ATP, 1.0; GTP, 0.1; KCl, 60.0; magnesium acetate 2, 1.5; spermine, 0.56; Tris-HCl (pH 7.6), 15.0; ribosomal protein, 1.86 mg; pH 5 protein, 1.80 mg; and [^{14}C]phenylalanine, 4.9 mμmoles (0.07 μc). Incubation was in an 0.5-ml volume for 30 min. When present, 2,4-DNP was 0.1–1.0 × 10^{-4} M; azide was 0.5–2.5 × 10^{-4} M. All ingredients except for radioactive phenylalanine were added to tubes kept on ice. The tubes were then warmed at 30° for 2 min and the [^{14}C]phenylalanine was added (uniformly labeled 110 mc/mmole). Incubation was for 30 min at 30° and was terminated by plunging the tubes in ice. Incubation mixture (0.1 ml) was pipetted into 1-in. square pieces of filter paper and the papers were immersed in 10% TCA and washed as described by Mans and Novelli (1960). The papers were suspended in 0.4% PPO–toluene and assayed for radioactivity in a Nuclear-Chicago Model 725 liquid scintillation spectrometer.

Amino Acid Pools. Batches (200 ml) of cells at a Klett reading of 100 were incubated anaerobically alone or with 10^{-4} M dinitrophenol or 2 × 10^{-4} M sodium azide. Samples were taken at 0, 20, and 60 min for each of the three incubations. The cells were removed by centrifugation and washed two times with fresh medium minus glucose, ammonium sulfate, and yeast extract. The pellets were suspended in 5 ml of water and disrupted by shaking with 18 g of glass beads (Superbrite glass beads, type 100-5005, 3M Co.) for 1 min in the cold in a Nossal vibrator. The beads were successively washed with small portions of water until a 25-ml total volume was obtained. After removing 1 ml for protein determination, 3 ml of water and 3 ml of 100% TCA (w/v) were added. The precipitate was washed two times with 5% TCA. All TCA extracts were pooled and after decomposing the TCA by autoclaving for 1 hr at 121°, the extracts were concentrated by evaporation to 5-ml volumes, and aliquots were analyzed on a Beckman Model 120 amino acid analyzer.

Sucrose Density Studies. A culture grown anaerobically to early log phase was incubated for 20 min with [^{14}C]leucine–isoleucine or [^{14}C]uracil in the presence and absence of inhibitors, and then iced, centrifuged, and washed two times with isotonic saline. The cells (5–6 g) were ground in a mortar with twice their weight of acid-washed sand, and then extracted with a double volume of 0.05 M Tris (pH 7.5)–0.005 M magnesium acetate buffer. The extract was centrifuged at 20,000*g* for 20 min. Supernatant fluid (1 ml) was layered on a 5–25% sucrose gradient and centrifuged for 3.5 hr at 25,000 rpm in a no. 25 Spinco head. The gradient was made by sequentially diluting a 13.2-ml portion of 25% sucrose with a 14.4-ml portion of 5%

sucrose in a double-chamber gradient maker (Britten and Roberts, 1960). Linearity of the gradient was confirmed by using methylene blue in the heavy solution. The tube was punctured at the bottom with a 21-gauge needle and successive 1-ml fractions were collected. These fractions were assayed for optical density in a Beckman Model DU spectrophotometer at 240, 260, and 280 mμ and for radioactivity as determined with Bray's solution (Bray, 1960) in a scintillation spectrometer.

Isotopic Materials. The [^{14}C]leucine, -proline, -valine, and -phenylalanine used in these studies had a specific activity of 12.2 μc of ^{14}C/μatom of carbon, were uniformly labeled, and were isolated from algae grown in $^{14}CO_2$ (Hendler, 1961). [2-^{14}C]Uracil (65 mc/mmole) was purchased from New England Nuclear Corp.

Measurement of TCA-Soluble 260-mμ Absorbing Material. An aliquot of the TCA-soluble material used for amino acid analysis as described above, from which the TCA was removed by heating at 121° in an autoclave, was examined in a Cary spectrophotometer Model no. 14M, and the amount of 260-mμ absorption was measured. 2,4-DNP and sodium azide at the concentrations used do not contribute to the 260-mμ absorption.

Double-Isotope Experiments. Growth medium (1 l.) containing 0.5 mc of [^{32}P]phosphate was inoculated with 0.3 ml of a fully grown culture and incubated anaerobically overnight at 30°. When optical density measured with a no. 54 filter was 100, incubations were set up with 0.5 μc of [^{14}C]uracil/ml. To one incubation flask 10^{-2} M 2,4-DNP was added to 10^{-4} M final concentration. These flasks were sealed and incubated at 30°.

To study uracil incorporation, duplicate 0.1-ml samples were withdrawn at designated times and pipetted onto 1-in. square 3MM Whatman filter paper. These papers were put through TCA, ethanol–ether, and ether washes by the Mans and Novelli (1960) technique, one set being heated in 5% TCA and one not. The papers were then placed in 0.4% PPO–toluene and the radioactivity was determined in a Nuclear-Chicago liquid scintillation counter with double-isotope settings determined for ^{14}C–^{32}P by techniques previously described (Hendler, 1964). The difference between the radioactivities of the hot and cold trichloroacetic acid washed papers in PPO–toluene was taken as a measurement of [^{14}C]uracil and [^{32}P]phosphate incorporation into nucleic acid. The cold trichloroacetic acid washed papers (after radioactivity assay) were washed in chloroform and then heated in 5% TCA, reprocessed, and recounted in PPO–toluene to give a double check on the heated TCA papers. The specific activity (^{14}C/^{32}P counts per minute) indicated the [^{14}C]uracil incorporation into RNA while at the same time correcting for differences in cell density with the ^{32}P. Measurements of nucleotide pools and of the specific activities of UTP, UDP, and UMP pools from these cultures were carried out.

At various intervals 10 ml of these anaerobic cultures was removed, washed with isotonic saline, and resuspended in 2 ml of 10% TCA. The supernatant was extracted with 12 mg of acid-washed Norit A for 20 min. The charcoal was washed free of acid and the nucleotides were eluted with 50% ethanol–1% NH_4OH. Aliquots of 0.1 ml out of a final volume of 10 ml were placed onto 1-in. squares of 3MM Whatman filter paper, dried, and counted by liquid scintillation in 0.4% PPO–toluene for ^{32}P which gives a comparison of the total amount of the nucleotide pool in control and dinitrophenol-treated yeast.

The charcoal eluate was concentrated and chromatographed for 17 hr in acid Leloir solvent pH 3.5 (1.0 M ammonium acetate, pH 3.5, three parts; 95% ethanol, seven parts). The mono-, di-, and triphosphate nucleotides were separated by chromatography, eluted, aliquots were counted by liquid scintillation as before, and the remaining samples were electrophoresed in 0.05 M ammonium formate (pH 3.5) for 30 min at 4200 v and 260 ma in order to separate the uracil-containing nucleotides (UMP, UDP, and UTP) from their companion nucleotides. The spots were located by ultraviolet light, cut out, and assayed for radioactivity by liquid scintillation in 0.4% PPO–toluene under conditions determined for ^{32}P–^{14}C (Hendler, 1964).

Dinitrophenol and Azide Uptake Studies. Cells were grown anaerobically to mid-log phase and divided into 100-ml aliquots. Control samples were chilled on ice while the experimental groups were incubated for 10 min at 30° in the presence of 10^{-4} or 10^{-5} M 2,4-DNP or 10^{-4} M potassium azide. The potassium azide was 95% ^{15}N terminally labeled and analyzed to be 35% atom % excess of ^{15}N by mass spectrometry. Glucose-deficient cells were obtained by collecting cells at mid-log phase and resuspending them in glucose-deficient medium for 12 hr at 30° before use. After incubation, samples were chilled, collected, and either directly sonicated to 99% cell destruction or washed two times with various solutions prior to sonication treatment. A 105,000*g* supernatant solution was prepared from the sonicated suspension. Control samples of cells were dried in a vacuum over at 80° for 48 hr after the washes in order to determine dry weight. Some of the dried samples were further washed with various solutions and then extracted with chloroform–methanol (2:1) to see if any more DNP could be recovered. [^{14}C]Dextran was added to various samples to determine the quantities of extracellular water and inhibitors were carried forward into various fractions. Aliquots of the 2,4-DNP solutions were read at 365 mμ in a Beckman Model DU spectrophotometer and the quantity was calculated from an empirically determined curve.

The azide content was determined by measuring atom per cent excess by mass spectrometry. Each set of saline washes or 105,000*g* supernatant solution of the sonicates was treated identically as follows.

Sodium azide (1 ml of 2×10^{-2} M) was added to each tube to provide sufficient N_2 pressure for measurement in the mass spectrometer. The solution was dried and the tube was evacuated to 10–20-μ pressure,

TABLE I: Inhibitory Effects of DNP and Azide in Anaerobic Yeast.[a]

Inhibitor (M)	Cell Growth		α-Glucosidase Induction	[14C]Leucine Inc into Protein		[14C]-Uracil Inc into RNA
	Lag	Mid Log		Non-growing	Log Growth	Log Growth
			DNP			
10^{-4}	17	14		6	18	31
5.0×10^{-5}	60		40			
10^{-5}	71	95				
			Azide			
2.5×10^{-4}	0.7	27				
2.0×10^{-4}				12	9	17
5.0×10^{-5}	30	67	55			

[a] All numbers represent per cent of the control. Cell growth as determined by turbidimetric methods was measured over a 7-hr growth period from the initial inoculum and for 1 hr after addition of uncouplers to mid-log yeast. α-Glucosidase induction was measured as the amount of growth during the early growth phase (up to 4–5 hr after inoculation). [14C]Leucine and [14C]uracil incorporation was measured by the membrane filter technique of Britten *et al.* (1955).

then closed off by means of a vacuum-tight stopcock. The tube was heated at 365° in a sand bath for 10 min to decompose the azide to N_2 gas. It was essential to suspend the reacted vessels in liquid N_2 for 1 hr prior to analysis and to have a trap chilled in liquid nitrogen between the sample and machine. This procedure reduced the background due to pyrolytic decomposition products. The atom per cent excess of ^{15}N was determined and from this the moles of azide in each fraction were calculated. To one control tube of each set 10 or 20 μl of 10^{-2} M $K^{15}N_3$ was added before the sodium azide in order to test recovery and for calibration of the instrument.

Results

Effects on Protein and RNA Synthesis. The effect of 2,4-DNP and azide on the rate of protein synthesis was measured by three different methods under growing and/or nongrowing conditions. The rate of growth was measured turbidimetrically from lag phase through log phase and during mid-log growth and converted to milligrams dry weight from empirically determined curves. The rate of induction of α-glucosidase was determined by measuring the rate of growth in cells forced to adapt to a maltose medium thus encompassing nongrowing (lag phase) and growing phase (mid-log phase). The final method of determining the rate of protein synthesis was to measure the rate of [14C]leucine, [14C]valine, and [14C]proline incorporation into yeast protein over a short period in mid-log phase of growth and in a nongrowing phase. Correcting for milligrams dry weight did not alter these measurements because of the small percentage of change of dry weight in the control over the time interval used. Table I shows that 2,4-DNP and azide inhibited protein synthesis in anaerobically incubated yeast as measured by all three methods under varying growth conditions with good agreement between the methods at corresponding concentrations. The same results were obtained for [14C]valine and [14C]proline as for the [14C]leucine shown.

The inhibition of RNA synthesis as measured by the rate of [14C]uracil incorporation into RNA is also shown in Table I. As in the amino acid studies, correction for the milligram dry weight did not alter the inhibition because of the small percentage change over the time interval used. In this latter experiment it was determined that the specific activity of the [14C]UTP pool, in the case of the inhibited cells, was as high or higher than the control cells so that RNA synthesis was truly inhibited. In Figure 1 it is shown that the inhibition of RNA synthesis occurs at a time when the pool of radioactivity in the acid-soluble components is equal to or greater than was the case for the control. Therefore, with pool sizes the same or greater than controls, the data to be discussed below, the decreased incorporation of [14C]uracil into the cellular RNA represents a true inhibition of synthesis. It is interesting that the initial rate of labeling of the acid-soluble pool is decreased in the inhibited cases, but this delay is completely overcome by 5–10 min whereas inhibition of incorporation continues (Figure 1).

Effects on ATP. During phases of pronounced inhibition, the ATP pools per milligram dry weight were directly measured and found to be the same or higher for the inhibited cells compared to the controls (Table II). Actual ATP values, after conversion from micrograms per milligram dry weight cells, ranged from 55 to 19 μmoles/100 g wet weight of cells as the organism passed from early-log phase to late-log phase of growth. These levels are in good agreement with

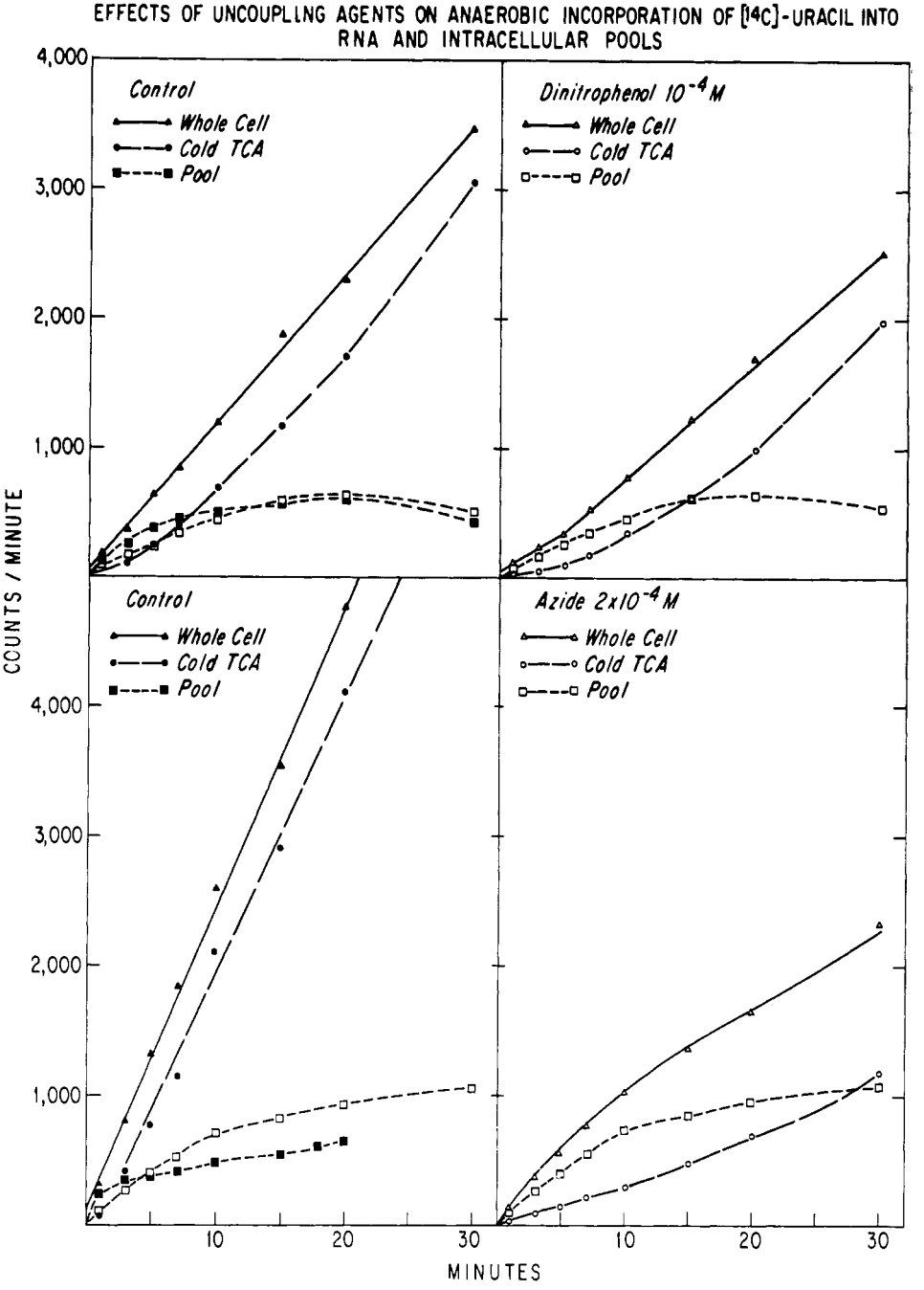

FIGURE 1: Effect of 10^{-4} M 2,4-DNP and 2×10^{-4} M azide on incorporation of [^{14}C]uracil into RNA and intracellular precursor pools by anaerobically grown log-phase yeast. [^{14}C]Uracil (0.5 μc) (65 mc/mmole) per milliliter of culture was used. At each time point, 2 ml of culture was filtered on a membrane filter (0.45-μ pore size) and 2 ml put into 0.2 ml of 100% trichloroacetic acid in an ice bath which was later filtered. Filters were glued to stainless-steel-ribbed planchets and counted in a low-background gas-flow counter. The difference between whole cells and TCA precipitate gives the pool size. Pools from 2,4-DNP- and azide-treated cells are shown superimposed on controls for ease of comparison.

the value previously reported by Schmitz (1954) for yeast. Several investigators (Wu and Racker, 1958; Honig and Rabinovitz, 1966; Hempling, 1966) have reported that 2,4-DNP does not affect ATP levels in ascites tumor cells and suggest that increased glycolysis compensates for any decrease due to ATPase stimulation by the poison.

Effects on the in Vitro Amino Acid Incorporation

TABLE II: Effect of Uncouplers on Growth and ATP Concentration.[a]

Min after Addn of Uncoupler	10^{-4} M 2,4-DNP		2.5×10^{-4} M Azide		5×10^{-5} M Azide	
	Growth	ATP Concn	Growth	ATP Concn	Growth	ATP Concn
20	27.5	104	15	88	45	102
40	18.0	131	15	113	49	101
60	14.0	151	27	115	67	146

[a] All values are expressed as per cent of the controls. Growth measurements, determined turbidimetrically, represent the time during the interval from the previous to the indicated time point. ATP concentrations were originaly determined as micrograms of ATP per milligram dry weight.

TABLE III: Effects of 2,4-Dinitrophenol and Azide on Amino Acid Pools of Exponentially Growing Yeast.

		20 min after Addition					60 min after Addition				
	Zero-Time Control	Control	Azide (2×10^{-4} M)		2,4-DNP (10^{-4} M)		Control	Azide (2×10^{-4} M)		2,4-DNP (10^{-4} M)	
Amino Acid	(mμ-moles/mg of protein)	(mμ-moles/mg of protein)	(mμ-moles/mg of protein)	% Control	(mμ-moles/mg of protein)	% Control	(mμ-moles/mg of protein)	(mμ-moles/mg of protein)	% Control	(mμ-moles/mg of protein)	% Control
Lysine	98.0	89.0	177.0	199	79.0	89	56.0	91.0	162	60	107
Histidine	7.0	5.4	16.0	296	8.8	163	4.1	6.3	153	3.8	93
Ammonia	90.0	72.0	139.0	193	72.0	100	43.0	60.0	140	54.0	126
Arginine	36.0	36.0	67.0	186	28.0	78	19.0	25.0	132	13.0	68
Aspartic acid	21.0	20.0	38.0	190	34.0	170	13.0	25.0	192	26.0	200
Threonine	35.0	28.0	35.0	125	24.0	86	16.0	8.1	51	13.0	81
Serine	1.4	1.2	2.0	167	9.7	808	8.6	7.5	87	8.6	100
Glutamic acid	45.0	37.0	77.0	208	37.0	100	25.0	32.0	128	33.0	132
Proline	11.0	8.1	11.0	136	6.5	80	4.9			4.0	82
Glycine	43.0	46.0	85.0	185	48.0	104	34.0	31.0	91	39.0	115
Alanine	16.0	16.0	70.0	438	32.0	200	11.0	39.0	355	55.0	500
Half-cystine	3.4	10.0	10.3	103	39.0	390	10.1			9.2	91
Valine	7.5	5.3	12.0	226	4.2	79	1.8	4.7	261	4.6	256
Methionine	2.9	1.4	1.5	107	1.6	114	0.64			0.44	69
Isoleucine	7.7	4.6	5.8	126	3.1	68	2.0			2.5	125
Leucine	4.9	3.9	5.4	138	1.9	49	2.0			1.8	90
Tyrosine	0.62	0.62	2.6	419	1.0	161				0.62	
Phenylalanine	0.96	0.86	4.6	534	3.2	372				2.3	

System. A standard ribosomal amino acid incorporating system was prepared from the yeast. This system was ATP and pH 5 fraction dependent and poly U responsive but totally insensitive to the effects of either 2,4-DNP or azide. The response of this system to stimulation of phenylalanine incorporation by poly U was likewise unaffected by the poisons.

Effects on Amino Acid Formation from Glucose. Table III shows that there was no marked depletion in the pool for any amino acid as a result of treatment with either poison. On the contrary, most pools were enlarged. The leucine pool after 20 min of exposure to 2,4-DNP was the lowest, 49% compared to the control, but the same pool measured at 60 min was 90% of the control. At 20 min, in the presence of azide, leucine was 138% of the control. Therefore, inhibition of protein synthesis by these inhibitors is not related to a specific block in the synthesis of leucine or any other amino acid from glucose.

Effects on [^{14}C]Leucine and [^{14}C]Uracil Distribution among Ribosomal Populations. Figure 2 illustrates the distribution of [^{14}C]leucine among the ribosomal and polysomal components of the cellular supernatant fluid after a 20-min labeling period and the pattern

of the inhibition resulting from exposure to 2,4-DNP or azide. The pattern obtained for the control in terms of relative distribution of 260-mμ absorbing material in the monosome and polysome regions is the same as that reported by Marcus *et al.* (1965). The area under the polysome region on the basis of 260-mμ absorption compared to the area under the monosome region is decreased 50% by 2,4-DNP and 69% by azide relative to the ratio of these areas in the control. The distribution of radioactivity was at a maximum in the region of the 80S ribosome peak. Specific activities were at their lowest in the polysomal regions and increased gradually with decreasing density through the monosomal region in all cases. 2,4-DNP and azide inhibition of labeling, as determined by specific activity was distributed uniformly throughout the polysomal and monosomal regions.

A sucrose density centrifugation pattern for cytoplasmic supernatant fluids from cells labeled anaerobically for 20 min with [^{14}C]uracil is shown in Figure 3. As was true in the experiment shown in Figure 2, the relative amount of 260-mμ absorbing material in the heavier density regions was decreased in the cases of 2,4-DNP and azide treatment as compared to the monosome region. The predominant localization of rapidly labeled RNA was in the 80S ribosomal region as opposed to the heavier regions. The specific activities of the ribosomal classes increased from the heavier to the lighter density regions. Inhibition of incorporation by the poisons was quite uniform throughout the polysomal and monosomal regions as illustrated by the specific activities. It is most interesting that the peaks for specific activities occurred in a lighter density region than the 80S ribosomal location. This could perhaps be ribosomal precursor (Kitazume *et al.*, 1963) or the newly hypothesized informosome type of particle (Spirin, 1967).

Effects on Nucleotides. The endogenous pool sizes for the sum of all the nucleotides as measured by 260-mμ absorption of TCA-soluble material was not diminished during the period of inhibition caused by 2,4-DNP or azide. On the contrary, the aggregate pool size seemed in general to be slightly increased. This would be consistent with a block in the utilization of nucleotides for synthetic processes. This is confirmed by showing that ^{32}P content of the total nucleotide pool is unaffected by up to 45 min of exposure to 2,4-DNP. After separating the nucleotides into mono-, di-, and triphosphate groups by chromatography, it was found that the amount of ^{32}P incorporated into the respective groups in the 2,4-DNP-treated cells was equal to or greater than the control, indicating normal formation of each nucleotide group. When [^{14}C]uracil was

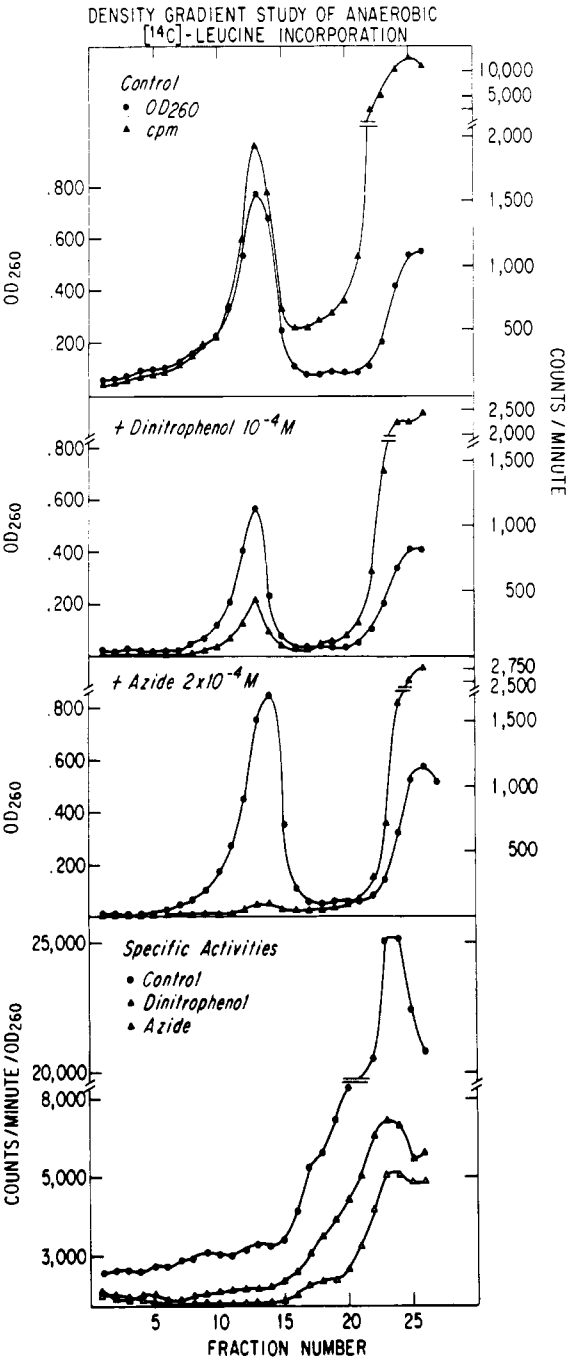

FIGURE 2: Effects of 2,4-DNP and azide on [^{14}C]leucine incorporation into 260-mμ absorbing material of anaerobically grown yeast. Poisons and 0.1 μc of [^{14}C]leucine/ml of culture were added to the culture at OD_{540} of 100 and it was incubated anaerobically for 20 min at 30°, then chilled, centrifuged, washed, ground with acid-washed sand, and extracted with 0.05 M Tris-HCl plus 0.005 M Mg-acetate buffer (pH 7.5). A 20,000*g* supernatant extract was layered on a 5–25% sucrose gradient and centrifuged for 3.5 hr at 25,000*g* in no. 25 Spinco rotor head. Samples of 1 ml were collected and assayed for 260-mμ absorption and radioactivity. The lower numbers represent the high-density region. The sensitivity of labeling the monosome region (fractions 12–15) to azide and 2,4-DNP shows that metabolically incorporated radioactive material is depicted in these gradients.

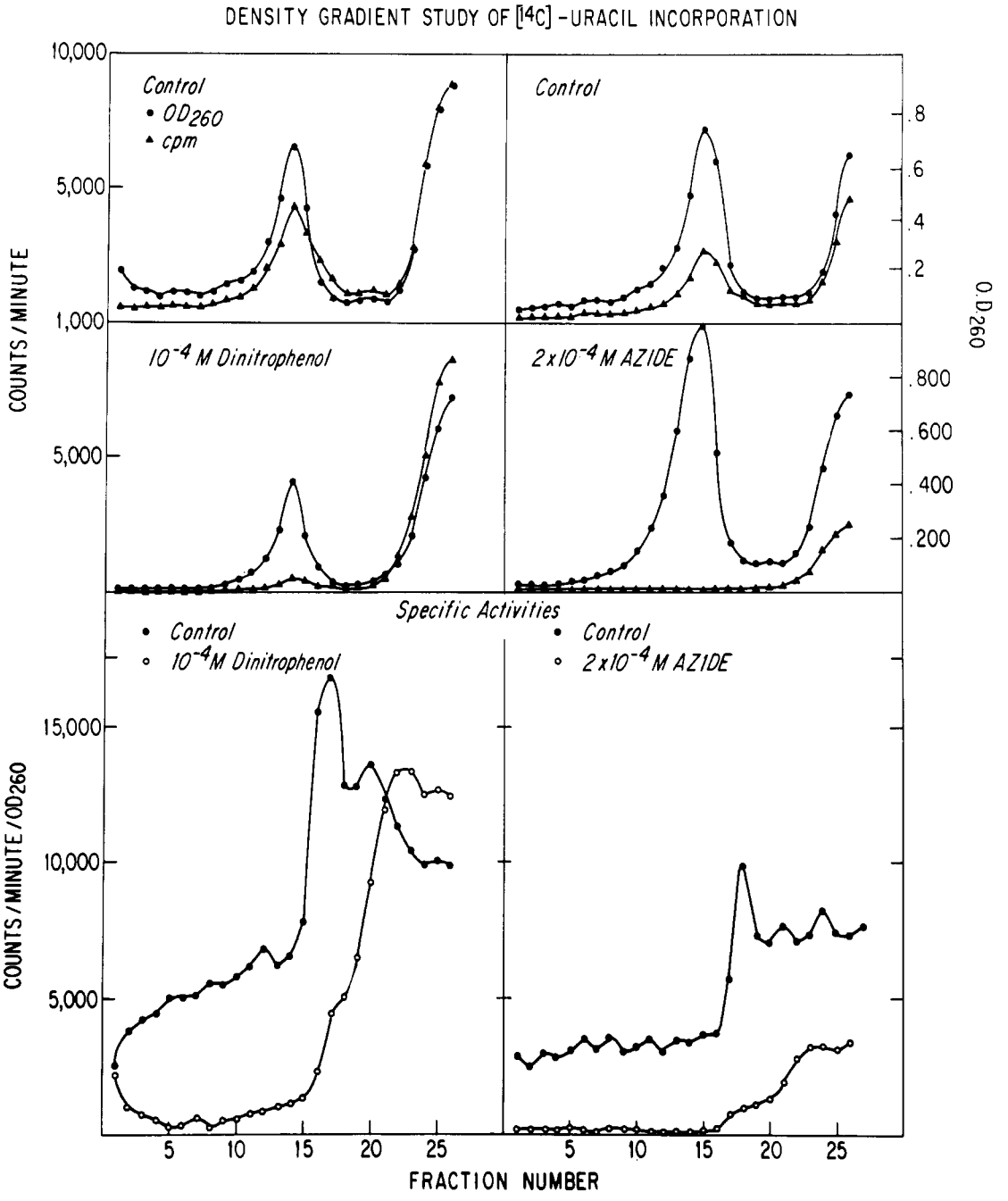

FIGURE 3: Density gradient study of [^{14}C]uracil incorporation into 260-mμ absorbing material. To 200 ml of log-phase yeast was added 2 μc of [2-^{14}C]uracil (sp act. 65 mc/mmole) and 2,4-DNP or azide was added as indicated.

added to cells grown on [^{32}P]phosphate from the inoculum stage, its incorporation into UMP, UDP, and UTP as determined by specific activities of the nucleotides purified from the previously mentioned nucleotide pools was unaffected by 10^{-4} M 2,4-DNP. In fact, the specific activities of the uridylic acid pools were elevated in the presence of 2,4-DNP. This interesting finding could be explained if the slightly elevated pools mentioned above decreased the flow of unlabeled precursor coming from glucose without affecting the incorporation of external radioactive uracil into nucleotides. This interpretation is consistent with the observation that asparatic acid transcarbamylase, a key enzyme in pyrimidine biosynthesis, in *Escherichia coli*, is very sensitive to inhibition and repression by pyrimidine nucleotides (Yates and Pardee, 1956). The above data indicate that in the presence of the inhibitors, GTP and CTP levels must be normal, since the total nucleotide pools, the triphosphate pools and ATP levels are normal and UTP formation is unimpaired.

Effects on DNA-Dependent RNA Polymerase. It was found that DNA-dependent RNA polymerase, isolated from anaerobically grown cells (Chamberlain and Berg, 1962), is insensitive to concentrations of 2,4-DNP and azide 10–100 times greater than those used in our experiments.

Discussion

The data presented in this study raise questions as to the validity of current concepts of the energetics of protein and RNA synthesis in the intact cell. 2,4-DNP and azide are chemically very dissimilar, yet both of these agents in very low concentrations will uncouple oxidation from phosphorylation. Dinitrophenol is believed to cause the hydrolysis or decomposition of an early nonphosphorylated high-energy intermediate (Slater and Tager, 1963; Vitols and Linnane, 1961; Utter *et al.*, 1958). Azide inhibits cytochrome oxidase and also blocks an energy conversion somewhere between the stage affected by 2,4-DNP and the final product of oxidative phosphorylation, ATP. Azide will also inhibit the splitting of ATP induced by 2,4-DNP (Dawkins *et al.*, 1960). Although azide can elicit ATPase activity, this activity is small compared to that of 2,4-DNP, is transient and completely overshadowed by its inhibitory effect on ATP breakdown after 20 min, and requires concentrations much higher than those used in our studies (Myers and Slater, 1957). Therefore, under the conditions employed in our experiments, 2,4-DNP can be thought of as an ATPase-stimulatory substance and azide as non-ATPase stimulating. Whereas azide is generally inhibitory to heme-containing enzymes, 2,4-DNP in the concentrations at which it uncouples oxidative phosphorylation is much more specific. At very much greater concentrations, 2,4-DNP has been reported to affect the DNA-dependent RNA polymerase in *E. coli* (Gros *et al.*, 1963).

The reactions of protein and RNA synthesis have been studied for the most part in *in vitro* systems in which energy is supplied and utilized in the form of ATP. Under anaerobic conditions in whole cells with ATP produced by glycolysis, uncouplers of oxidative phosphorylation would not be expected to inhibit protein and RNA synthesis if ATP is used directly. The fact that these uncouplers do inhibit these synthetic reactions is amply proved in the literature and in the studies reported here. The inhibition does not depend on growth and is readily observed under nongrowing conditions. We have shown that during such inhibition, high levels of ATP are present. Furthermore we have shown that a normal and adequate supply of precursors for protein and RNA synthesis is maintained in inhibited cells. Neither 2,4-DNP nor azide inhibited any of the reactions of an *in vitro* amino acid incorporating system, namely amino acid activation, aminoacyl-tRNA formation, binding of mRNA, reading of mRNA, and peptide-bond formation when ATP was the energy source. In addition, the activity of DNA-dependent RNA polymerase was not impaired by the poisons when ATP was the energy source. The incorporation of exogenous uracil into UTP was similarly uninhibited. Inhibition as revealed by the distribution of specific radioactivities upon density gradient centrifugation following both amino acid and uracil labeling was evenly distributed throughout the gradient and not specifically directed against the polysome class of ribosomes. Although there was a decrease in the fraction of ribosomes in the heavier density regions, the bulk of highly labeled ribosomes in the untreated cells was encountered in the 80S regions. Inhibition occurred instantaneously and did not increase with continued incubation. Since at the time of addition of the poisons, the cells were amply supplied with ribosomes, messengers, and all other necessary forms of RNA, it would appear that the inhibition of protein synthesis is an independent event and is not a result of a primary inhibition in the formation of some key form of RNA leading to a progressive deterioration of its supply. The question is why, in the presence of the necessary ATP, do two chemically dissimilar uncouplers of oxidative phosphorylation immediately prevent protein and RNA synthesis? Several possible explanations may be considered. (1) There may be some other essential reaction, perhaps yet undiscovered, which is rate limiting *in vivo*, and which is sensitive to both DNP and azide, yet untested in the present study. (2) There may be compartments in the cell where protein and RNA synthesis go on with greatest efficiency where ATP levels are decreased due to the ATPase effect of 2,4-DNP. For this explanation to be valid, it would require that azide behave atypically since it would be functioning predominantly as an ATPase of the same or greater efficiency than 2,4-DNP. (3) The cell may have the ability to transport and build up very great concentrations of DNP and azide either in the cytoplasm in general or in localized compartments where essential reactions of protein and RNA synthesis occur. The latter possibility cannot be adequately tested. It would seem to be unlikely, however, that the cell would maintain such compartments for protein and RNA synthesis and also for the energy-independent concentration of chemically unrelated but both highly destructive agents.

The former possibility was directly tested for both 2,4-DNP and azide. Table IV shows the cells do have an affinity for 2,4-DNP. But the uncoupler is loosely held and easily removed by washing the unbroken cells. Even in the total absence of energy an apparent six- to ninefold concentration is calculated by assuming that all bound 2,4-DNP is inside the cells. Since it is impossible to build such a concentration gradient in the absence of energy, the uncoupler must be present in a bound form and near the outer surface of the cells. A very similar type of affinity of uncoupling phenols for proteins in general and mitochondria in particular has been described by Weinbach and Garbus (1965). These authors think that such binding is important in the uncoupling action of these agents. Whereas inhibitions of protein synthesis were observed with 10^{-5} M 2,4-DNP, the maximum possible average of intracellular

TABLE IV: Uptake of 2,4-Dinitrophenol in Yeast.[a]

	Treated with 10^{-5} M 2,4-DNP Expt (μmoles/g dry wt)			Treated with 10^{-4} M 2,4-DNP Expt (μmoles/g dry wt)			
	1	2	3	1	2	3	4
			Unbroken Cells				
1st wash	0.19	0.25	0.00	6.25	6.67	1.35	1.80
2nd wash	0.01	0.18	0.00	2.22	2.47	0.24	0.37
3rd wash	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			Destroyed Cells				
Released by extraction	...	0.00[c]	...	0.00[b]	0.00[b]	0.00[c]	0.00[c]
Total	0.20	0.43	0.00	8.47	9.14	1.59	2.16
Max total molarity	10^{-4}	2×10^{-4}	0.00	4.4×10^{-3}	4.3×10^{-3}	6.6×10^{-4}	8.6×10^{-4}

[a] In expt 1 and 2 incubations were for 10 min at 30° with glucose present. Experiments 3 and 4 employed glucose-starved cells, the former kept at 0° during treatment and the latter incubated for 10 min at 30°. Maximum total molarity was calculated on the basis of all of the inhibitor being dissolved in the intracellular water. In these experiments washes were done with water. Similar results were obtained when 0.15 M NaCl–0.1 M Tris (pH 7.4) was used. [b] Determined by breaking a parallel culture with ultrasonication and extracting with water. [c] Cells directly extracted with $CHCl_3$–CH_3OH (2:1).

concentration that could be formed in this case is less than concentrations found to be without effect in the *in vitro* systems tested. Table V presents the first data obtained for azide binding. Like 2,4-DNP, this uncoupler is also bound by the cells and quite unexpectedly to the same extent on a micromolar basis. Although approximately 75% of the bound azide is readily removed by washing the unbroken cells, the remainder is held more tightly and released only after extensive ultrasonic treatment of the cells.

TABLE V: Uptake of Azide by Yeast Cells.[a]

	Experiment (μmoles/g dry wt)[b]		
	1	2	3
	Unbroken Cells		
1st wash	...	2.44	2.75
2nd wash	0.80	1.22	0.89
	Destroyed Cells		
Released by extraction	1.21	1.22	1.38
Total	...	4.88	5.02
Max total molarity	...	4×10^{-3}	3.9×10^{-3}

[a] All cells were incubated for 10 min at 30° in the presence of glucose. Washes were carried out with 0.15 M NaCl. Cells were broken by ultrasonication and extracted with 0.15 M saline. Maximum total molarity was calculated on the basis of all the inhibitor being dissolved in the intracellular water. [b] Treated with 10^{-4} M.

(4) Another possibility is that in the intact cell a high-energy intermediate(s) similar to or identical with those of oxidative phosphorylation is used as an energy source for protein and RNA synthesis and not ATP directly. Bronk (1963) using rat liver mitochondria with succinate as energy source, showed that leucine incorporation into protein was virtually eliminated by antimycin A and 2,4-DNP but was not inhibited by oligomycin at concentrations which block ATP formation. Furthermore, tetraiodothyroacetic acid, a potent inhibitor of phosphorylation, virtually doubled the rate of leucine incorporation. Although Kroon (1963a,b, 1964) made similar observations with beef heart and rat liver mitochondria regarding the insensitivity of amino acid incorporation to oligomycin he was more cautious in his interpretation. He pointed out that the magnitude of incorporation of amino acids by mitochondrial protein in the systems studied was quite low. If ATP levels were not limiting then the small level of oligomycin-insensitive ATP production might be sufficient to drive the amino acid incorporation. He also cautioned that secondary effects such as high energy-induced uptake of certain ions may be the cause of enhanced protein synthesis. Campbell *et al.* (1966) reexamined the question in a system with a much higher level of protein synthesis, namely rat brain mitochondria, and found the same indication of a

possible coupling for the systems of electron transport and protein synthesis as did Bronk.

Hendler (1961) and Kovac and Istenesova (1964), have suggested that the energy of oxidative phosphorylation may be used directly for protein synthesis of intact cells. Since oxidative phosphorylation as well as protein synthesis (Hendler, 1965) and perhaps RNA synthesis (Suit, 1962; Abrams *et al.*, 1964) occur in membrane-bound systems, a cytostructural unit may exist which couples these processes without the use of ATP. An alternative to this is that ATP is formed either by mitochondria or glycolysis and then split by membrane-bound ATPases into a high-energy form(s) similar to that of oxidative phosphorylation and used directly for these two and possibly other membrane-bound synthetic processes. In either case, under anaerobic conditions energy would be fed back at the expense of ATP formed from glycolysis and uncouplers of oxidative phosphorylation would be able to disturb this flow of energy from ATP. Evidence for the existence of such a system is the report of Schatz (1965) who demonstrated the presence of an oligomycin-inhibited ATPase in membrane fractions of anaerobic yeast.

The results of these studies strongly suggest the use of a high-energy intermediate(s) similar to or identical with those of oxidative phosphorylation as energy for protein and RNA synthesis. A more direct demonstration will depend on isolating a cell organelle capable of an efficient coupling of these two systems. Such studies are currently underway.

Acknowledgments

The acellular amino acid incorporation studies were performed in the laboratory of Dr. M. V. Simpson, Department of Biochemistry, Dartmouth School of Medicine. The authors are indebted to Dr. Simpson for his consideration and help. The mass spectrometric analysis was carried out by William Comstock, National Cancer Institute. The authors appreciate his expert assistance in this problem.

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