Intracellular Mechanisms for the Control of Respiration and Biosynthesis in Mycobacteria

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This is actually an exact parallel of the situation I encountered in my graduate work in comparative biochemistry. My courses in microbial biochemistry and metabolism were taught by a wonderful research professor, whose name ranks very high in this field. He recognized the lack of available firm information on bacterial metabolism and would very often delve deeply into a complex topic by first informing the students that, although such and such a cycle or reaction was undoubtedly present in bacteria, there was insufficient evidence to secure the hypothesis. Accordingly, he would say, we will look at the biochemical details of this process, which has been thoroughly investigated in, for example, liver or muscle or sea urchin eggs. The approach, although novel, worked. We rapidly learned his meaning of careful critical research despite the source of the cell or extract. In this roundabout fashion I

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would like to inform Dr. Long now that I appreciate the difficulties inherent in this conference and hope that some of the information we have gained from our work on *Mycobacterium tuberculosis* will either answer questions or perhaps stimulate thinking about problems in leprosy.

It seems to me, an outsider looking in, that lack of progress in a field can be due to any one or to a multiplicity of causes; for example, the problem is too difficult for present technology, or we are investigating the wrong organism, or we are relatively stupid, and so on. Perhaps from this conference we will gain some insight on this aspect of the problem. In this vein there is one final word. Ideas, like bacteria, do not grow in a sterile environment. Contributions are made only when there is a rapid and free flow of ideas and concepts between participants. I hope this conference maintains the requisite informality which is the precursor of the progress.

The work that I shall now present is the product of many man years of intensive research around the question, how is the biosynthetic machine that we call *M. tuberculosis* regulated? By what pathways does it make itself? Can we control its metabolism?

I would like to give credit to several of my associates, past and present, whose incisive experimental work has led to these results. The work on allosteric control mechanisms is that of Dr. Abraham Worcel. Mr. Frank Lornitzo has singlehandedly carried out the arduous work on the inhibition of disaccharide formation, and Drs. Andre Piérard, Yasuhiro Kanemasa and Takashi Kusaka have taken the problem of biosynthesis of mycobacterial lipids from the ordinary to the extraordinary.

I would like to begin the research discussion with some general ideas of control systems that operate in the growth and development of cells. One of the major differences between unicellular and multicellular organisms is that the former respond to intracellular control systems only, while the latter respond to both intra- and intercellular control systems. If we limit our consideration of metabolic control systems to those systems that are responsible for the metabolism and energy production of the cell, and have nothing to do directly with the reproduction of the cell, then there are several well-known possibilities for the sites of action of such control systems:

(1) A metabolite acts to turn off the enzyme responsible for its formation. This is routine product inhibition; the control is on the rate of an enzyme-catalyzed reaction.

(2) A metabolite acts on an enzyme several steps removed from the formation of the metabolite. This is feed-back control.

(3) A metabolite acts on a specific enzyme-forming system to block or alternatively to permit the synthesis of a specific enzyme. This is repression-depression.

(4) A relatively new form of metabolic control, i.e., a form that has only recently been recognized, is that type of metabolic control exhibited by metabolites unrelated to either substrates or product but active in controlling the rate of an enzyme-catalyzed reaction. We are now dealing with molecules that exhibit allosterism—acting on another site.

I shall discuss some of our recent work on the metabolism of *M. tuberculosis* and divide the discussion into three parts. First, we will consider an enzyme system vital to the economy of the cell and two metabolites that demonstrate both allosteric activation and allosteric inhibition of this complex. Second, we shall consider an enzyme of importance in the formation of disaccharides and, probably, in the formation of liposaccharides. This enzyme is under the control of an inhibitor unrelated to either product or substrate, and the inhibitor is, in turn, under control of another metabolite. Third, we shall consider an enzyme complex responsible for the biosynthesis of complex lipids. We have not demonstrated any metabolic control over this system; we hope that one may be discovered.

The system that is under allosteric control is, surprisingly, the membrane-asso-

ciate NADH[2] oxidase system. I say surprisingly because we were indeed surprised to find this effect in a particulate system. Since there are many indications of a conformational change associated with the action of an allosteric activator we had assumed that allosterism would be restricted to soluble systems rather than to the complex mosaic of a terminal oxidase.

The reaction carried out by the particulate oxidase is the complete oxidation of NADH by oxygen. The energy associated with the transfer of electrons from substrate through the flavoprotein dehydrogenases, through the quinone intermediate, through the cytochrome pigments and, finally, to oxygen, is retained by the cells in the form of high energy phosphate and used for the multitude of chemical reactions necessary for the biosynthesis of cellular components.

Cells are ground mechanically and the cellular debris is removed. The particles are isolated by differential centrifugation and are washed several times. Under the electron microscope the particles appear to be spherical and have an average diameter of 80 Å. The particles tend to form clusters, generally 3 or 4 of the basic particles to a cluster, but sometimes going up into much larger numbers. The only enzymatic activity so far discovered associated with these particles is the oxidation of NADH; no oxidation of succinate α-ketoglutarate or pyruvate has been found([5]).

The data of Figure 1 present the evidence that the pyridine nucleotides are oxidized via the cytochrome chain. It may be seen that reduction peaks are found in the area of cytochromes *a*, *b*, and *c*. The rate of emergence of each of the visible reduction peaks suggests that the electrons are transferred first to flavin, then to cytochrome *b*, then to cytochrome *c*, then to cytochrome *a*, and finally to oxygen. Both qualitative and quantitative differences exist between the cytochrome content of these particles and the cytochrome content of mitochondria derived from animal tissues. First, I should like to stress the extremely low concentration of the cytochrome pigments in this particle and, concomitantly, the very low rate of oxygen uptake carried out by these particles in the presence of substrate.

Second, there is the important difference that the *c* type cytochrome is actually cytochrome c_1 and not the soluble cytochrome *c*. The *b* cytochrome is quite atypical in that it is present both in the particles and in a soluble form. The *a* type cytochrome is atypical in that it is unaffected by cyanide or azide. Carbon monoxide is the only inhibitor that we have yet found effective in this system.

It is implicit from what has gone before that we have assumed that the electron transfer process associated with the oxidation of the NADH yields the necessary energy for the functioning of the cell. This energy yield is through the process of oxidative phosphorylation, i.e., the binding of inorganic phosphate into high energy phosphate associated with the oxidation of substrate. For the past several years we have diligently searched for evidence of this process of oxidative phosphorylation; we have been uniformly unsuccessful in this search. It was during these investigations that we found the clue that indicated the presence of an activation and inhibition of the particulate oxidase that had been hitherto unsuspected([12]). The tracing in Figure 2 shows the result of a typical experiment indicating that something new had been found.

In this experiment the oxidation of NADH is followed by the disappearance of oxygen from solution; the method is amperometric. NADH is generated by the alcohol—alcohol dehydrogenase system; no disappearance of oxygen takes place until the last component of the system is added. Oxygen consumption then takes place at a relatively linear rate. About halfway through this experiment a small amount of AMP was added to the reaction vessel. It

[2]The following abbreviations are used: NAD and NADH (also generally referred to as DPN and DPNH), oxidized and reduced nicotinamide adenine dinucleotide; NADP and NADPH (also generally referred to as TPN and TPNH), oxidized and reduced nicotinamide adenine dinucleotide phosphate; CoA, acetylation coenzyme; AMP, ADP and ATP, adenosine 5′ -mono-, 5′ (pyro) -di-, and 5′ (pyro) - triphosphates; EDTA, ethylenediaminetetraacetate; Tris, Tris (hydroxymethyl) aminomethane.

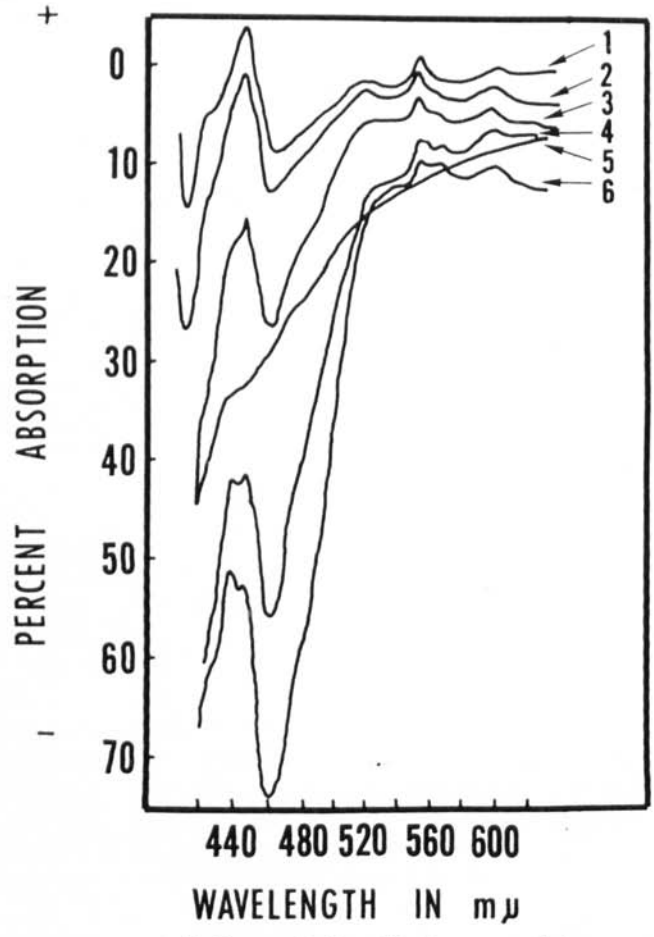

FIG. 1. Time course of the reduction of the Triton ×-100-activated NADH oxidase by NADH. The crude enzyme (20 mgm. protein/ml.) was reduced with NADH. Spectra were taken at 2 min. (1), 5 min. (2), 30 min. (3) and 60 min. (4). The cuvette was opened to the air (5), following which the enzyme was reduced with dithionite (6). The curves shown are transcriptions of the original data and are normalized at 600 mμ for convenience. The 600-mμ values are separated one from the other by 2% absorption to preserve clarity in this composite figure. (*From* Goldman, Wagner, Oda and Shug, *Biochim. Biophys. Acta* 73 (1963) 367).

will be seen that the rate of oxygen consumption increased by about 50 per cent. Upon further investigation of this effect, we discovered that the stimulation of the rate of oxygen uptake was highly specific; of all of the nucleoside mono-, di-, or triphosphates tested, only AMP showed this effect. Among the desoxynucleoside mono-, di-, and triphosphates tested, only desoxy-AMP had any effect on the system and this was only a small per cent of that shown by AMP itself. To our knowledge this type of effect had never been found in a particular enzyme. We were certain at this point that we did not have what is known as metabolic control, i.e., the control of the rate of oxygen uptake during the oxidation of NADH by the concentration of an ADP and inorganic phosphate. We felt that our lack of ability to demonstrate oxidative phosphorylation under standard conditions with these particles might be referable to the fact that the normal mechanism of metabolic control with these particles was not the one normally associated with, for example, mitochondrial particles. Further investigation of this effect of AMP on the particles indicated that we had a very powerful mechanism for the control of respiration in this organism.

I must digress slightly in order to make this point clear. Under ordinary laboratory conditions an enzyme is assayed in a totally artificial environment. Obviously, the assay system is contrived in a fashion to permit the enzyme to turn over at its maximum rate. It is perfectly obvious that in the cell these conditions do not obtain and that the enzyme is under various metabolic controls. One of the most obvious controls is that the substrate for an enzyme is never present in maximum amounts. In general, the substrate is present at about the concentration of the Michaelis constant, i.e., the concentration of substrate that permits half maximum velocity of the enzyme. At

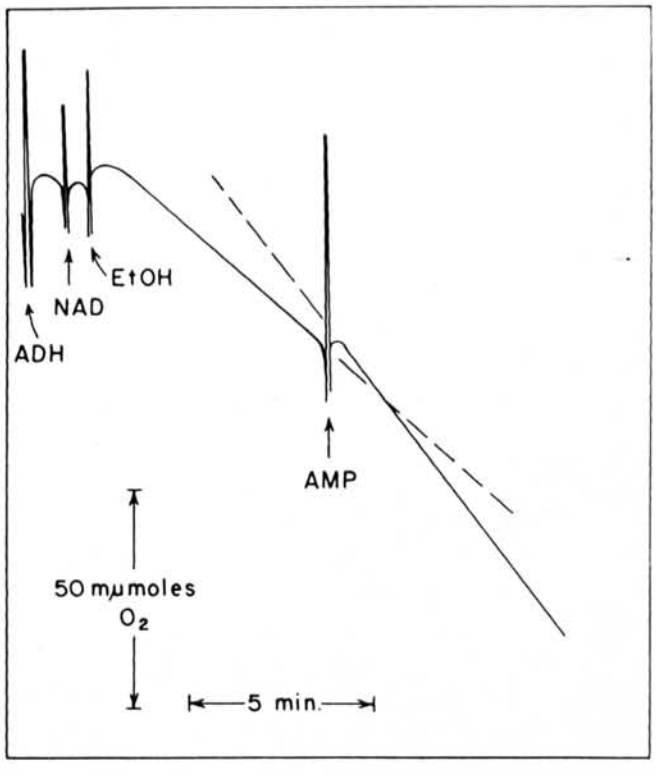

FIG. 2. Effect of AMP on the respiratory rate of washed particles of *M. tuberculosis*. NADH oxidation (oxygen uptake) was followed polarographically. The reaction mixture contained 200 μmoles of tris-acetate buffer, pH 7.5; 10 μmoles of $MgCl_2$; 0.10 mgm. of alcohol dehydrogenase; 2 μmoles of NAD; 100 μmoles of ethyl alcohol, and 0.5 to 1.0 mgm. of NADH oxidase protein in a final volume of 2.0 ml. Oxygen consumption was followed at 30°C with a vibrating platinum electrode. Additions to the polarographic cell were made as shown. (*From* Worcel, Goldman, and Cleland, *J. Biol. Chem.* **240**(1965) 3399).

this concentration the velocity of the enzyme will be roughly proportional to the concentration of substrate. While this control mechanism is of use it simply is not fine enough, since it does not provide for any *amplification* of a change in substrate concentration. This amplification must be provided by an alternate mechanism in which the rate of the enzyme reaction is non-linear with respect to the substrate concentration. This can be brought about only by modification in the mechanism of the enzyme-catalyzed reaction. The effect of the AMP concentration on the rate of substrate oxidation is shown in Figure 3. The linearity of the double reciprocal plot suggests that only one molecule of AMP is associated with the activation of the enzyme. In Figure 4 is shown the effect of substrate concentration and of the presence of NAD, the product of the reaction, on the rate of the reaction. The bottom curve (which is actually parabolic in shape in this double reciprocal plot) is the plot of the reaction in the absence of inhibitor; it is this curve that we are concerned with. The parabolic shape has been confirmed by a computer analysis of the data and suggests that two molecules of substrates are bound to the enzyme before one molecule of product can be formed. With the inactive, or unactivated enzyme the product causes a competitive type of inhibition since the maximum velocities are the same regardless of the presence of the inhibitor. The shape of the double reciprocal plot remains parabolic. From Figure 5 it may be concluded that following activation of the enzyme by AMP, there is fundamental change in the character of the oxidation of substrate. The double reciprocal plots are now linear and this linearity is unaffected by the presence of inhibitor. The linearity of the plot suggests that only one molecule of substrate is bound to the enzyme.

A composite of the mechanisms that we have suggested for the allosteric transitions is shown in Figure 6. I have already presented evidence that the native enzyme is activated by AMP to give an activated form; I have not presented the evidence that the reversion of the activated to the native form is catalyzed by NAD. In the

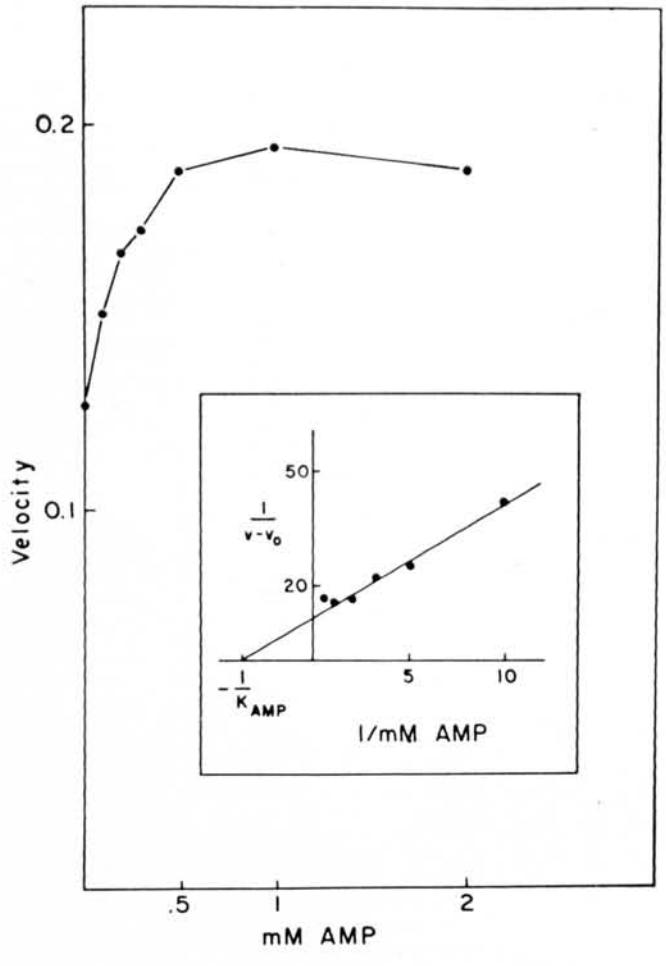

FIG. 3. Effect of AMP on the rate of NADH oxidation. The NADH oxidase spectrophotometric assay system contained 0.15 μmole of NADH and 100 μmoles of tris-acetate buffer of pH 7.5 in a final volume of 0.99 ml. After temperature equilibration for 3 minutes, 10 μl of the NADH oxidase preparation were added, and the change in absorbence at 340 mμ was followed. v is the absorbence change at 340 mμ per minute, $v_0 = v$ in the absence of AMP. (*From* Worcel, Goldman and Cleland, *J. Biol. Chem.* **240** (1965) 3399).

proposed mechanism the native enzyme first binds one molecule of substrate to give an activated intermediate which will not break down to yield product and enzyme. This is only accomplished by the addition of a second molecule of substrate to the activated intermediate. Both the native enzyme and the intermediate form of the enzyme bearing one molecule of substrate can bind inhibitor to give an unreactive inhibitor-enzyme or inhibitor-enzyme-substrate complex. This type of double substrate binding and dead end inhibition will yield the kinetic results discussed above. Upon activation of the enzyme by AMP the entire character of the reaction is changed. The activated enzyme now yields the active complex upon the addition of but one molecule of substrate. The active enzyme can also bind one molecule of inhibitor. However, this inhibitor-activated enzyme complex, exactly as can the activated enzyme itself, adds one molecule of substrate to give an activated complex, which in turn can break down to yield product and the inhibitor-enzyme complex again. Since the maximum velocities of the reaction are the same regardless of the concentration of inhibitor at infinite concentration of substrate, we must assume that k_3 and k_6 are identical. This is the simplest mechanism to account for the type of kinetic data derived from the experiments with the activated enzyme.

We have concluded that the particulate NADH oxidase of *M. tuberculosis* is under allosteric control. The control mechanism is actually the *ratio* of AMP to NAD rather than the concentration of either metabolite. It should be emphasized here that the concentration of AMP is dictated primarily by the action of the enzyme, adenylate kinase.

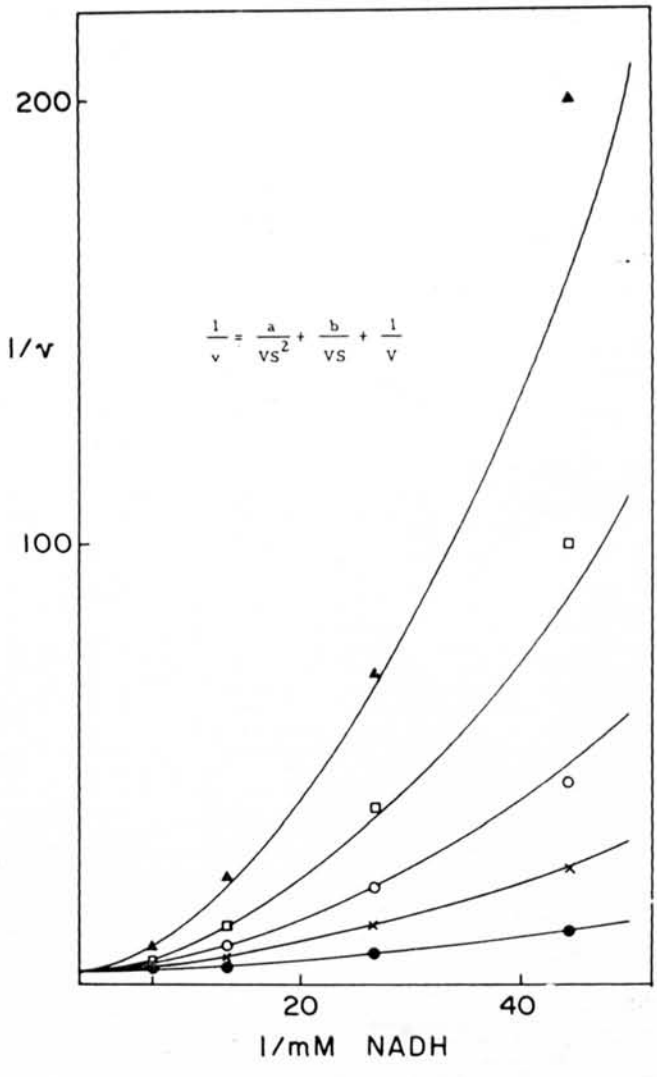

FIG. 4. NAD inhibition of the native NADH oxidase. The assay system employed was identical with that described in Figure 3 except that 0.352 mgm. of NADH oxidase protein was added to each incubation mixture. NAD additions to the basic reaction mixture were as follows: (•) no NAD; (x) 0.1 μmole; (○) 0.25 μmole; (□) 0.5 μmole; (▲) 1.0 μmole. The *points* are experimental; the *lines* are calculated from a computer fit of the data to the equation shown. (*From* Worcel, Goldman, and Cleland, *J. Biol. Chem.* **240** (1965) 3399).

The equilibrium of adenylate kinase is such that as the ADP is phosphorylated to yield ATP, the AMP concentration in the cell, normally just high enough to activate the NADH oxidase and oxidative phosphorylation, drops to a point where activation is not seen. The oxidase then comes under the control of the NAD concentration, thus reducing the oxidation rate until ATP is needed. In this event the utilization of ATP yields ADP and the adenylate kinase raises the AMP concentration to a level where activation takes place. Not only does the activated oxidase turn over faster, but it should be remembered that the activated oxidase is relatively insensitive to NAD. In short, the energy production of the cell is controlled by the energy needs of the cell.

I would like to turn to another type of control mechanism found in *M. tuberculosis*. For some time we have been concerned with the question of avirulence in the H37Ra strain. One characteristic of this strain is that while it produces mycolic acid[1] it does not produce cord factor[2]. The relationship between cord factor, tetralose-6, 6′-dimycolate, and trehalose is shown in Figure 7. The structure of cord factor was derived by Noll, Bloch and collaborators[3,10]. Trehalose is a nonreducing sugar characteristically produced by fungi; the pathway for the biosynthesis of trehalose was discovered by Cabib and Leloir[4]. Upon investigation[6] we noted that the H37Ra strain of *M. tuberculosis*, the avirulent strain, could not synthesize

TABLE 1. *Transglycosidase content of mycobacterial and yeast extracts.*

Strain	Preparation and age	Specific activity[a]	
		Crude extract[b]	Precipitated protein[c]
H37Ra, *M. tuberculosis*	Surface culture 28 day	0-65	0-35
"	Liquid culture, 10 day	80	50
H37Rv, *M. tuberculosis*	Surface culture 18 day	170	90
"	Liquid culture, 9 day	250	200
BCG, *M. tuberculosis*	Surface culture 20 day	100	70
Brewers yeast	Actitve fermentation	700-2,000	700

[a]mμmoles trehalose 6-P/mgm. protein/hr.
[b]The cell-free extract was centrifuged at 60-100,000 x g. The supernatant solution was dialyzed against 0.001*M* EDTA.
[c]The crude dialyzed extract was saturated with ammonium sulfate. The precipitate was recovered by centrifugation, dissolved in and dialyzed against 0.001*M* EDTA.
(*From* Goldman and Lornitzo, *J. Biol. Chem.* **237** (1962) 3332).

trehalose (Fig. 8). The sugar phosphates and nucleotides are separated by electrophoresis. That mycobacteria can synthesize trehalose is shown in Table 1. It is to be noted that the enzyme system is many times weaker than the corresponding enzyme system from yeast.

The variable specific activity of the H37Ra system was puzzling. While some variability in specific activity is commonly encountered in enzyme preparations, it is relatively infrequent that one finds a specific activity that varies from nothing to something. A series of experiments that

TABLE 2. *Inhibition of H37Rv transglycosidase by H37Ra extracts.*

Exp. no.	H37Rv protein		H37Ra protein added	UDP formed (mμmoles/hr)		Per cent inhibition
	Culture	Mgm.		Recovered	Expected	
1	18 day, surface	0.21		10.1		
		0.21	0.23	11.0	12.2	10
		0.21	0.68	4.6	14.2	68
			0.23	1.0		
			0.68	3.0		
2	18 day, surface	0.42		86		
		0.42	1.50	43	96	55
		0.42	1.50[a]	90	86	0
			1.50	8		
3	10 day liquid	0.10		6.6		
		0.20		15.5		
		0.10	0.40	29	28	0
		0.20	0.40	37	37	0
			0.40	21		

[a]Heated 2 min. at 100°C before addition.

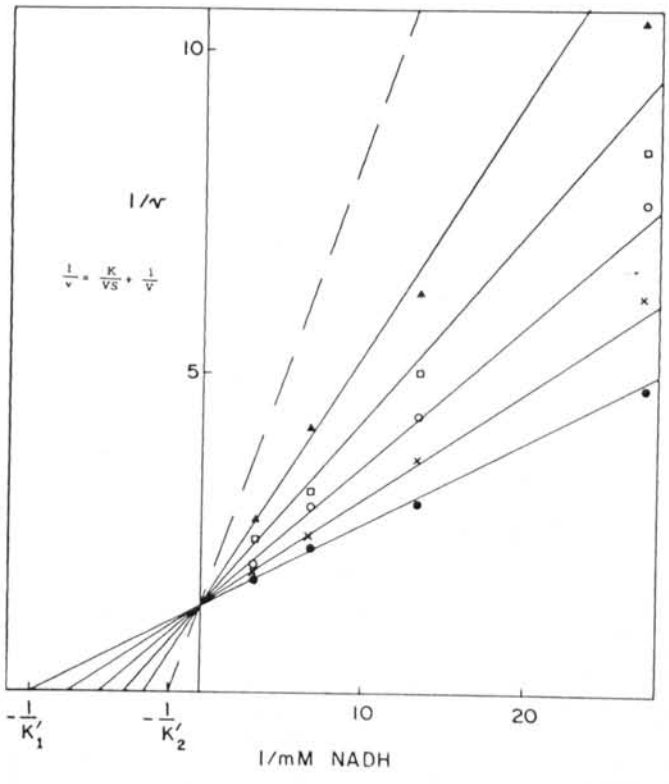

FIG. 5. NAD inhibition of the AMP-activated NADH oxidase. The conditions were identical with those described in Figure 3, except that a concentrated suspension of the NADH oxidase was preincubated at 0°C with a final AMP concentration of 2 mM. Aliquot samples of 20 μl. were withdrawn for the NADH oxidase assay. This dilution resulted in a final nucleotide concentration of 4×10^{-5}M and a protein concentration of 0.352 mgm. per ml. in the assay mixture. The spectrophotometric assay was used. The *points* are experimental; the *lines* are calculated from a computer fit of the data to the equation shown. The *dashed line* is calculated for [NAD] = ∞. (*From* Worcel, Goldman, and Cleland, *J. Biol. Chem.* **240** (1965) 3399).

will not be detailed here convinced us that we were not dealing with a simple enzyme system, but, rather, that the variability in the activity of the system that synthesizes trehalose was probably due to the presence of an inhibitor(6). Proof was obtained for the existence of such an inhibitor. Extracts of the virulent H37Rv strain of *M. tuberculosis* contain an active system for the formation of trehalose (Table 2). When the cell-free extract of the H37Ra strain is added to the Rv strain an inhibition of the production of trehalose is observed. A puzzling aspect of these experiments, an aspect whose significance we did not appreciate for some time, was that while the H37Ra extract could inhibit the formation of trehalose by the extract of the H37Rv strain when the extract was prepared from an 18-day surface culture of H37Rv, the same H37Ra protein could *not* inhibit the formation of trehalose when the extract was made from a 10-day old culture of H37Rv. Similar data were obtained

TABLE 3. *Inhibition of BCG transglycosidase by H37Ra extracts.*

Exp. no.	BCG protein		H37Ra protein added	UDP formed (mμmoles/hr)		Per cent inhibition
	Culture	Mgm.		Recovered	Expected	
1	21 day surface	0.15		3.8		
		0.15	0.15	7.1	8.0	11
		0.15	0.30	7.5	12.2	40
			0.15	4.2		
			0.30	8.4		
2	21 day surface	0.045		3.0		
		0.045	0.30	0.75	3.25	70
			0.30	0.25		

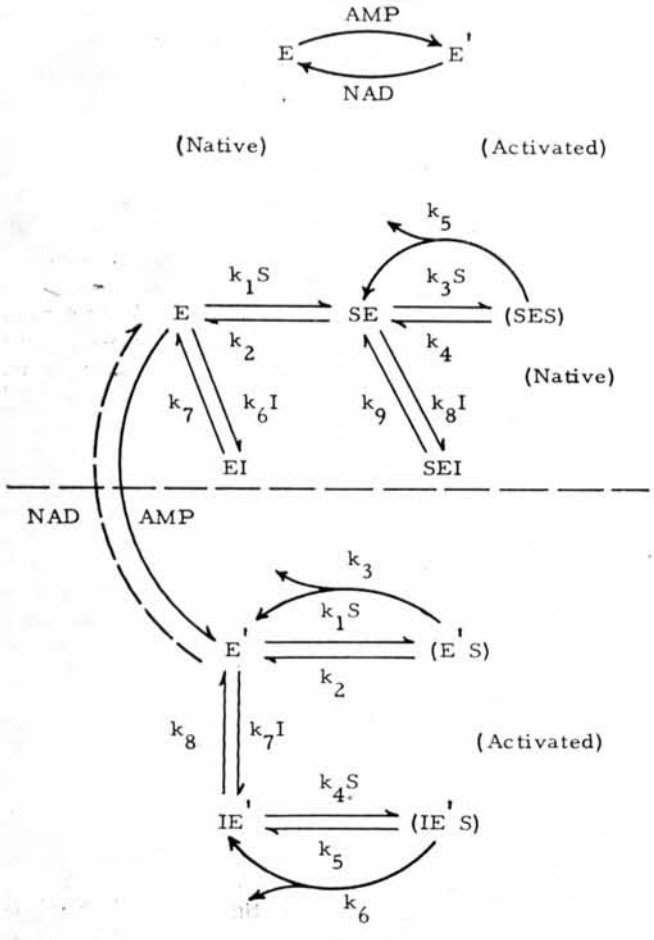

FIG. 6. Suggested mechanism for the allosteric transition and relaxation of the NADH oxidase of *M. tuberculosis*. **E** and **E'** are the "native" and "activated" forms of the enzyme, **S** is NADH, **I** is NAD and k_n is the kinetic constant for the indicated reaction.

using an extract of BCG (Table 3). About this time we recognized that the variability in specific activity of the trehalose-forming system of the avirulent strain was due to the variable content of this unknown inhibitory material in the extract of the cells([8]). This variability of inhibitor content was due to the variable destruction of the inhibitor. While this could be expressed as an activation of the trehalose-forming system, the activation was really a removal of an inhibitor. The nature of the inhibitory material was suggested by its behavior during dialysis (Table 4). If the crude cell-free extract of the H37Ra strain was prepared in EDTA buffer, the extract synthesized no trehalose. If, however, the cell-free extract was prepared in phosphate buffer or dialyzed against phosphate buffer, the extract showed activity in the trehalose-forming system. This activation phenomenon was not shown after the enzyme system had been partially purified; in other words, the partial purification of the enzyme system had eliminated the inhibitory material. We now had a partially purified enzyme and a crude inhibitor and the latter could be assayed against the former. The sensitivity of the inhibitor to phosphate, plus other experimental information, suggested that the inhibitor might be related to a polynucleotide. This suggestion was given additional strength by experiments in which the crude cell-free extract of H37Ra was fractionated with ammonium sulfate (Table 5). The enzyme system responsible for the formation of trehalose was spread across two fractions shown in Table 5 as R_2 and R_3. The enzyme which we suspected as being responsible for destruction of the inhibitor, polynucleotide phosphorylase, was also present in these two fractions. If fraction R_3 was refractionated, we were able to remove es-

TABLE 4. *Activation of the transglycosidase of H37Ra.*

Transglycosidase preparation	Treatment	Specific activity[a]
Crude extract	0.001 *M* EDTA, 2°, 6 days	0.0
"	0.001 *M* EDTA, dialyzed, 2°, 24 hrs	0.1
"	0.02 *M* PO_4^{3-}, 2°, 6 days	0.50
"	0.02 M PO_4^{3-}, dialyzed, 2°, 24 hrs	0.65
Partially purified	None, fresh preparation	6.3
"	0.002 M EDTA, dialyzed, 2°, 24 hrs	5.8
"	0.02 M PO_4^{3-}, dialyzed, 2°, 24 hrs	5.8

[a] mμMoles trehalose 6-P formed per minute per mgm.

(*From* Lornitzo and Goldman, *J. Biol. Chem.* **239** (1964) 2730).

sentially all of the trehalose-forming enzyme from the subfraction while retaining all of the polynucleotide phosphorylase activity. It is seen in the last column of the table that the inhibitor destruction capability of the various fractions went along with the polynucleotide phosphorylase. We were able to purify the inhibitor and showed that it was, indeed, a low molecular weight polyribonucleotide([8]). In addition to its rather unusual molecular makeup, it showed noncompetitive inhibitions of the trehalose-forming system (Fig. 9). This is a standard double reciprocal plot of velocity against substrate concentration in the presence of differing amounts of inhibitor. The fact that both the slopes of the lines and the y-intercepts vary is indicative of noncompetitive inhibition.

When we undertook the purification of the enzyme responsible for the formation of trehalose, we found that along with the purification of the enzyme there was a loss in sensitivity to the inhibitor([9]). What started out as a fully sensitive enzyme, i.e., an enzyme whose reaction could be

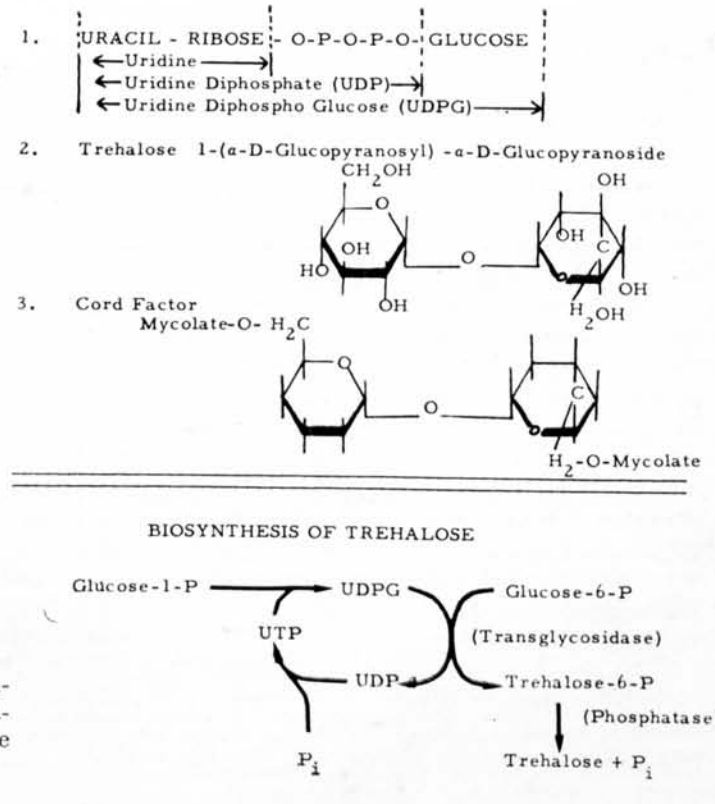

FIG. 7. The structural components of cord factor (trehalose-6, 6′-dimycolate and the biosynthesis of trehalose.

TABLE 5. *Correlation between inhibitor destruction and polynucleotide phosphorylase activity.*

Isoelectric precipitation		Transglycosidase activity[a]	Polynucleotide phosphorylase activity[b]	Inhibitor destruction (units/mgm. protein)
Fraction	pH			
R_2	5.1	3.5	2.2	0.42
R_3	4.65	2.9	2.2	0.41
Refractionation of R_3				
R_3R_1	5.0	2.5	2.0	0.41
R_3R_2	4.65	<0.1	1.8	0.55

Note: The crude cell-free extract was fractionated by ammonium sulfate followed by an isoelectric precipitation.

[a] $m\mu$Moles of trehalose 6-P formed/min/mgm. of protein.

[b] Units of nucleotide diphosphates formed from mycobacterial RNA.

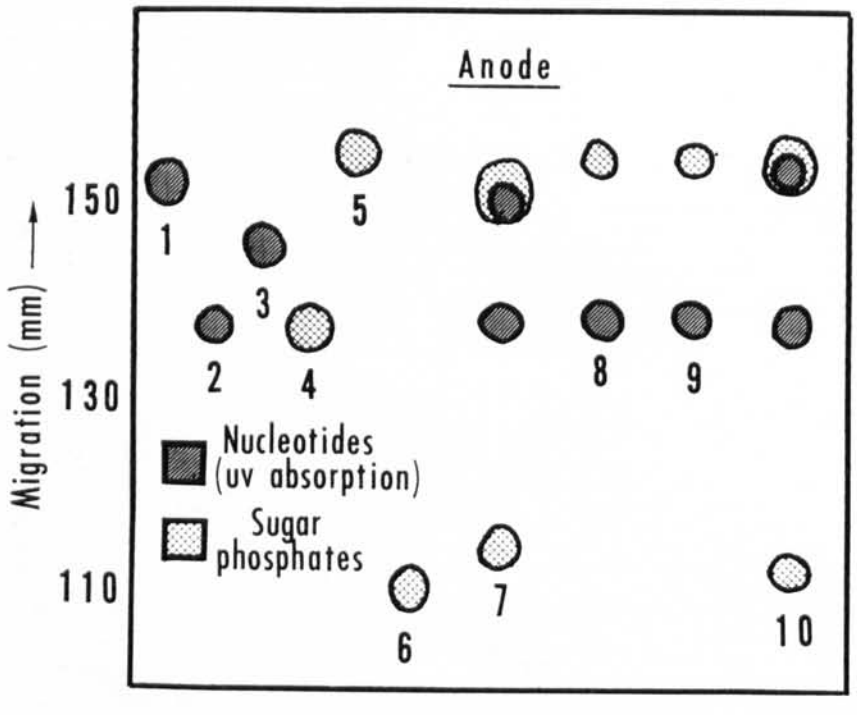

FIG. 8. Diagrammatic representation of electrophoretic determination of sugar phosphates and nucleotides. In the yeast and H37Ra transglycosidase systems the crude or partially purified cell-free extract is incubated with glucose-6-phosphate, UDPG, buffer and Mg^{++}. The protein is removed, and the sugar phosphates, nucleotides and nucleotide sugars are recovered and separated by electrophoresis in 0.2 M borate buffer (pH 9.7) on filter paper. See reference ([6]) for details. Under the described conditions the components are as follows: **1**, UDP; **2**, UDP-glucose; **3**, UTP; **4**, glucose-1-P; **5**, glucose-6-P; **6**, trehalose-6-P; **7**, yeast transglycosidase system; **8**, H37Ra extract extract tested for transglycosidase activity, complete system; **9**, same as **8** but lacking added glucose-6-P; **10**, yeast transglycosidase system to which H37Ra extract has been added; *uv*, ultraviolet. (*From* Goldman, and Lornitzo, *J. Biol. Chem.* 237 (1962) (3332).

blocked by the addition of the inhibitor, was rapidly converted to an enzyme that was completely insensitive to the presence of this inhibitor. After considerable experimentation we found that this conversion was reversible, and by appropriate manipulation we were able to convert the insensitive form of the enzyme back to the inhibitor-sensitive form of the enzyme (Table 6). What we have called here transglycosidase A is sensitive to the inhibitor that we have already purified and has been given the trivial name Mycoribnin. The transglycosidase B is the form that is insensitive to the inhibitor. It may be seen that as the purification of the enzyme proceeds the ratio of insensitive to sensitive goes from 10 to 0.05. However, desalting the enzyme preparation restores the ability of the inhibitor to block the reaction. Experimentation showed that we were removing neither a cofactor nor a metal ion, but only bicarbonate([9]). The ability of low levels of bicarbonate to prevent the inhibitory action of our purified inhibitor is shown in Figure 10. In the upper curve, the enzyme is treated with a known amount of inhibitor and the apparent inhibitor concentration is recorded on the vertical axis. The experiment is then duplicated with the one change that small amounts of bicarbonate

TABLE 6. *Reversible conversion of transglycosidase* A *to transglycosidase* B.[a]

Fractionation step	Purification over previous step	Ratio A/B
Crude extract	1	10
First $(NH_4)_2SO_4$	4	0.25
Second $(NH_4)_2SO_4$	1.25	0.05
Desalt over Sephadex G-50[b]	1	1.5
Chromatography on Sephadex G-200[c]	4	4

[a] *A*-form is sensitive to Mycoribnin.
B-form is insensitive to Mycoribnin.

[b] Equilibrated with 0.01*M* Tris-acetate—0.01*M* $(NH_4)_2SO_4$—0.002*M* Mg $(OAc)_2$.

[c] Eluted with 0.01*M* Tris-acetate-0.01*M* $(NH_4)_2SO_4$-0.002*M* Mg $(OAc)_2$.

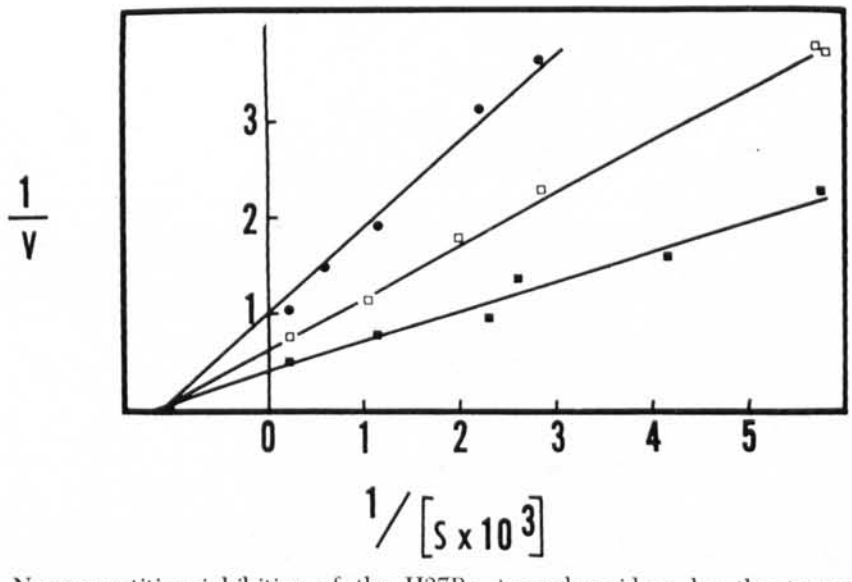

FIG. 9. Noncompetitive inhibition of the H37Ra transglycosidase by the transglycosidase inhibitor. Each reaction mixture contained 0.30 mgm. of crystalline bovine serum albumin (dialyzed), 0.030 μmole of NADH, 0.2 μmole of phosphenol pyruvate, 20 μl of a solution containing a 1:100 dilution of lactic dehydrogenase and a 1:20 dilution of pyruvate kinase, 3.5 μmoles of $MgCl_2$, 1.5 μmoles of EDTA, 1.0 μmoles of glucose-6-phosphate and 120 units of transglycosidase (specific activity of 300) and UDP-glucose (S) and transglycosidase inhibitor as shown. ■ — ■, no inhibitor; □ — □, 30 units of inhibitor; • — •, 60 units of inhibitor. Velocity is expressed as change in absorbence at 340 mμ due to the utilization of NADH in the pyruvate-lactate reaction. (*From* Goldman and Lornitzo, *J. Biol. Chem.* **237** (1962) 3332).

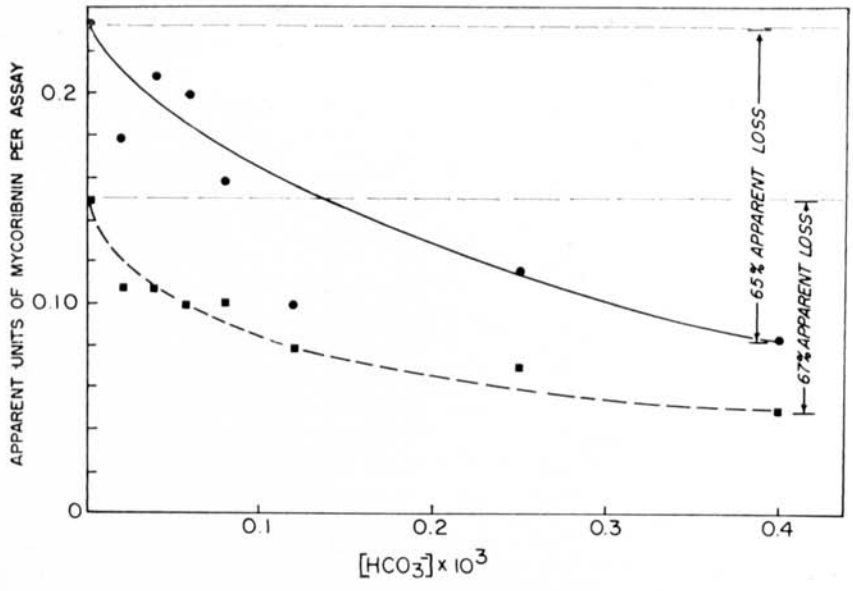

FIG. 10. Effect of bicarbonate concentration on the inhibition of the H37Ra transglucosylase by Mycoribnin. The assay system is substantially that described in the legend for Figure 9 except that bicarbonate was added, as shown, along with partially purified Mycoribnin as follows: • — •, 0.25 units; ■ — ■, 0.13 units. 1.0 unit of transglucosylase was used in each experiment. In these experiments 1 unit of enzyme is that amount of protein which catalyzes the formation of 1.0 mμmole of trehalose-6-phosphate in one minute under standard conditions. (*From* Lornitzo and Goldman, *J. Bact.* **89** (1965) 1086).

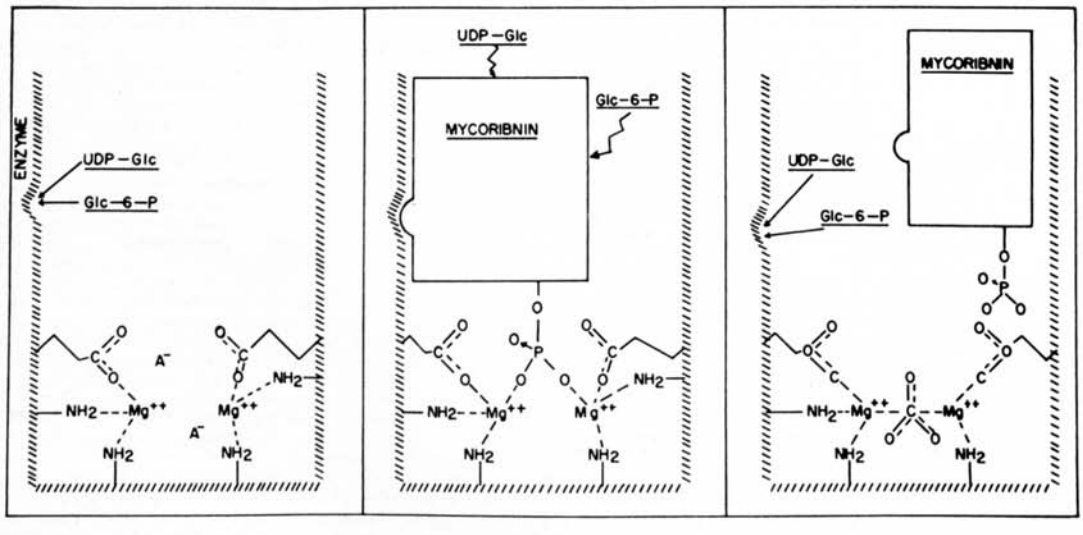

FIG. 11. Proposed mechanism for the action of Mycoribnin on the transglucosylase and for the reversible action of bicarbonate on the system. See text for details.

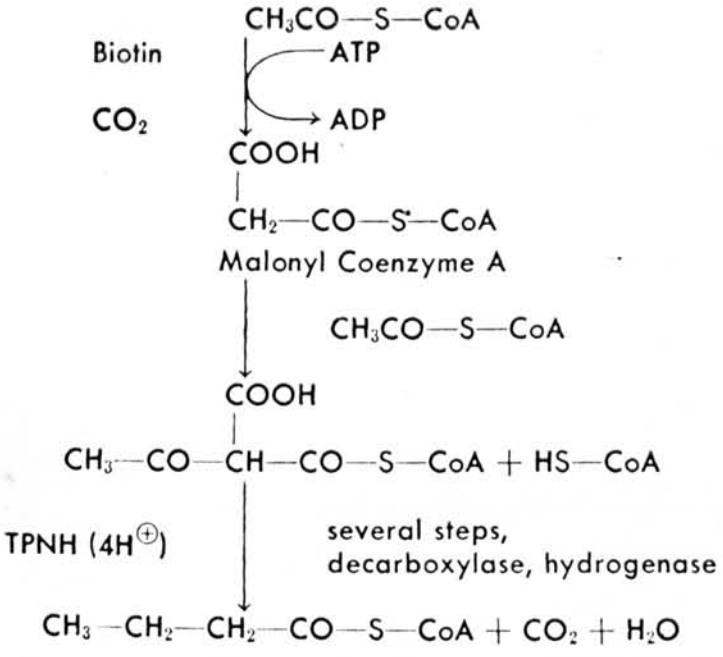

FIG. 12. Idealized version of the presently accepted mechanism for the formation of acyl CoA's from acetyl CoA and malonyl CoA.

are added to the reaction mixture. It is quite apparent that the addition of bicarbonate prevents the expression of inhibitory activity. The lower curve is the same experimental series except that the amount of initial inhibitor was smaller.

A remarkable feature of this bicarbonate effect was that it enabled us finally to explain our inability to show inhibition by the cell-free extract of the yeast trehalose-forming system as well as the trehalose-forming system from young virulent bacteria([6]). By carrying out the purification of the trehalose-forming enzyme from both yeast and young virulent bacteria under conditions where the bicarbonate concentration was kept very low, we were able to obtain enzyme preparations that were quite sensitive to the inhibitor. Addition of small amounts of bicarbonate to the enzyme completely reversed this sensitivity and the enzyme reverted back to the usual insensitive form([9]). These experiments indicate that as the bacteria age they probably change their overall metabolic direction to yield larger concentrations of intracellular bicarbonate either by actual bicarbonate production or by a change in the intracellular pH.

To summarize this portion of the discus-

TABLE 7. *Effect of octanoate on fatty acid synthesis from acetate by the soluble protein fraction from H37Ra.*

Addition	Amount	Acetate incorporated[a]
None		7.0
NADH	1.6 μmoles	21.0
$KHCO_3$	20 μmoles	14.2
NADH + $KHCO_3$		87
Octanoate	0.5 μmoles	60
Octanoate + NADH		320

Note: Each reaction mixture contained 100 μmoles of phosphate buffer (pH 7.0) 5 μmoles of ATP, 0.25 μmole of CoASH, 2.5 μmoles of $MnCl_2$, 8 μmoles of glutathione, 1.6 μmoles of NADPH, 2 μmoles of acetate-1-^{14}C (4.7×10^4 counts/min.) and 3 mgm. of protein. The final volume was 1.0 ml.; incubation was at 28°C for 1 hour.

[a] μMoles per hour per 10 mgm. of protein.

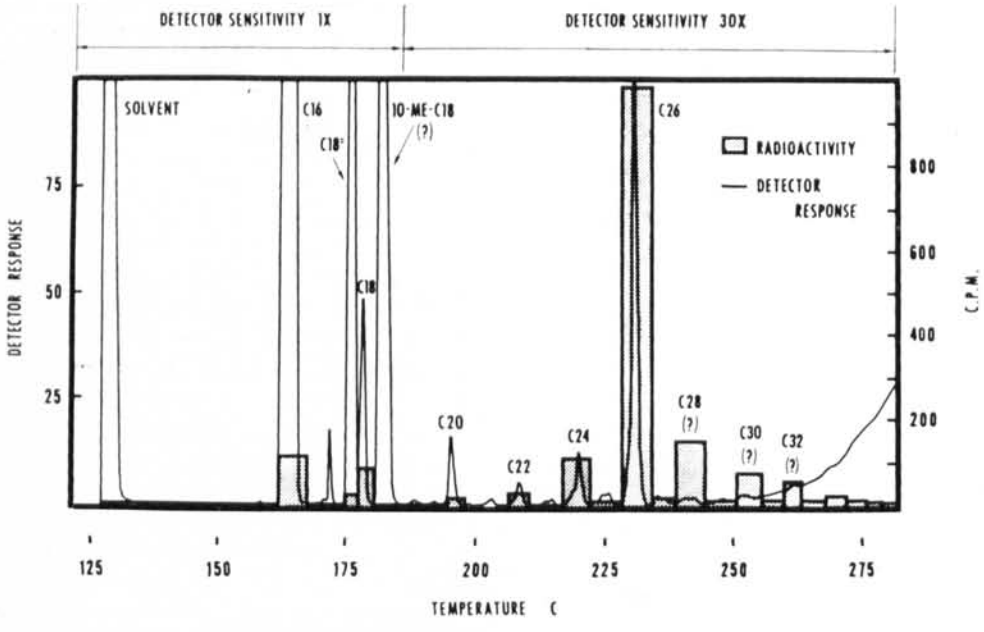

FIG. 13. Incorporation of malonate-2-^{14}C into long chain fatty acids. The reaction mixture contained 100 μmoles of phosphate buffer (pH 7.0), 0.25 μmole of CoA, 3 μmoles of ATP, 0.8 μmole of NADP, 2.5 μmoles of isocitrate, 3 μmoles of Mn^{++}, 8 μmoles of glutathione, 2 μmoles of malonate-2-^{14}C (2×10^5 counts/min.) and 10 mgm. of crude enzyme. The final volume was 1.00 ml. After 2 hours of incubation at 38°C the reaction was stopped and lipids saponified with alcoholic KOH. After acidification the fatty acids were extracted with petroleum ether and methylated with BF_3. Separation of the methyl esters was accomplished with a Barber-Coleman gas-liquid chromatograph. The stationary phase was ethylene glycol isophthalate (1.5%, by weight), on Gas-Chrom P; column length was 4 ft. The column bath temperature was programmed linearly at 3°C per minute. Total radioactivity of each sample, as counts per minute (c.p.m.), is shown by the height of the shaded areas; the time (temperature) interval over which a sample was collected is shown by the width of the shaded areas. (*From* Piérard and Goldman, *Arch. Biochem. Biophys.* **100** (1963) 56).

TABLE 8. *Effect of added fatty acids on the synthesis of long-chain fatty acids from acetate.*

Addition	Acetate incorporated (mμmoles)
None	19
Hexanoate	45
Octanoate	79
Decanoate	41
Palmitate	21

Note: Each reaction mixture contained 100 μmoles of phosphate buffer (pH 7.0), 5 μmoles of ATP, 0.25 μmole of CoASH, 2.5 μmoles of glutathione, 0.8 μmole of NADPH, 0.8 μmole of NADH, 2 μmoles of (1-^{14}C) acetate (4×10^5 counts per min), 0.5 μmole of fatty acid as indicated and 6 mgm. of protein (crude cell-free extract) in a final volume of 1.0 ml.

(*From* Kanemasa and Goldman, *Biochim. Biophys. Acta* **98** (1965) 476).

sion: we have found, in avirulent mycobacteria, a polyribonucleotide that specifically inhibits the formation of a disaccharide, which, in turn, is required for the formation of a toxic end product. The complexity of the system was increased by the discovery that the inhibitor itself was under the control of the simple metabolite, bicarbonate.

A suggested mechanism for this overall effect is shown in Figure 11. Certain experiments, not detailed here, have suggested that bicarbonate is exerting its effect at a point close to but not identical with the site at which Mycoribnin acts upon the enzyme. The first diagram in the figure shows our concept of the two substrates reacting at the enzyme surface. The structural and ionic specificity of Mycoribnin permits it to

move into position and act as a non-competitive inhibitor of the enzyme system since it is bound to the backbone of the protein chain. This binding would be through one of the several phosphate groups of Mycoribnin. Bicarbonate, on the other hand, can insert itself into and be bound at the same point along the protein chain that normally accepts the phosphate group of Mycoribnin. Without the firm point of attachment, Mycoribnin is ineffective as an inhibitor.

It is apparent, therefore, that the inhibition of this enzyme is a complex affair residing at two different metabolic levels. The unraveling of such a mechanism may provide clues as to the possible roads of interference in the metabolism of the infecting organism.

The third portion of this discussion on metabolic control points in the mycobacteria is, frankly, wishful thinking. It is evident that one of the major metabolic deviations of the mycobacteria from the normal metabolism of host cells is in the bacterial production of complex lipids. A case in point is the formation by the mycobacteria of mycolic acid, which is a necessary intermediate in the formation of cord factor. The biosynthesis of complex lipids by cell-free extracts of mycobacteria has been under investigation in our laboratory for some time. If one looks for it one can find the enzyme system for the synthesis of saturated long chain fatty acids from acetyl CoA. The present view of the mechanism of the first steps of this reaction, as worked out in several laboratories, is shown in Figure 12. The 4-carbon intermediate acetyl CoA is further elongated through malonyl CoA. The enzyme system responsible for the formation of long chain fatty acids has been isolated and investigated([11]). The results shown in Figure 13 summarize much of our work on this system. In this investigation the substrate was malonyl CoA formed from malonic acid, ATP, and CoA. The data shown here are a composite of the counting and gas chromatographic data. It is seen clearly here that malonyl CoA is incorporated into long chain fatty acids, predominantly into C_{26}. I indicated before that if you looked for it you would find this system. From a philosophic point of view it seemed to us unlikely that the mycobacteria would form enormously

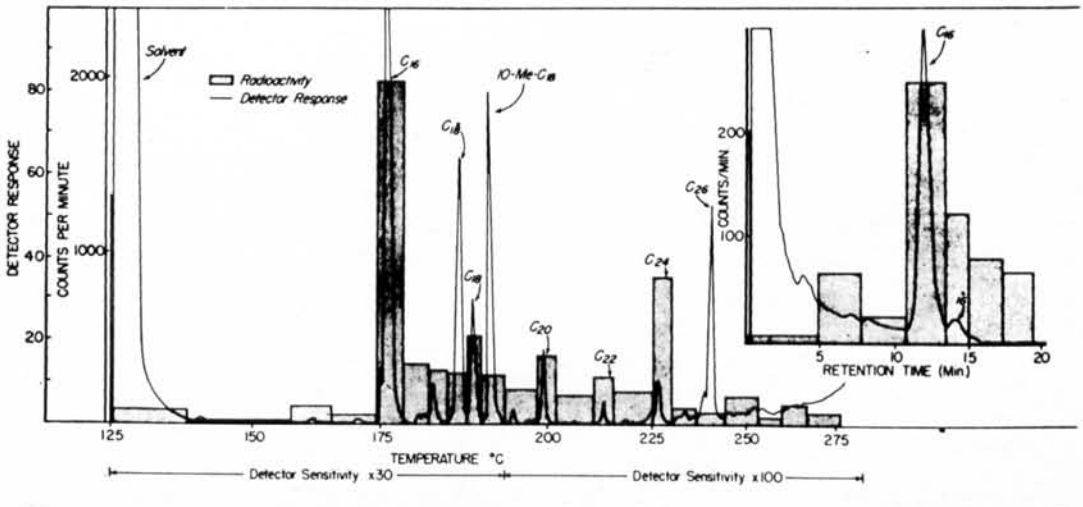

FIG. 14. Chromatography of the fatty acids synthesized from octanoyl CoA by the partially purified fatty acid system of *M. tuberculosis*. The reaction mixture contained 200 μmoles of phosphate buffer (pH 7.0), 4 μmoles of Mn^{++}, 8 μmoles of NADH, 10 μmoles of glutathione, 10 μmoles of ATP, 1.4 μmoles of (1-^{14}C)-octanoyl CoA (3.2×10^5 counts per min.) and 4.5 mgm. of protein. The final volume was 2.0 ml. Fatty acids were saponified, extracted, methylated and analyzed by the method detailed in the legend of Figure 13. The inset figure shows the isothermal (163°) rechromatography of the methyl C_{16} peak collected from the first chromatography. (*From* Kanemasa and Goldman, *Biochim, Biophys. Acta*, **98** (1965) 476.

TABLE 9. *Requirements for the incorporation of octanoyl CoA into long-chain fatty acids by the fractionated system.*

Omission	Addition	Octanoyl CoA incorporated (mμmoles)
None		46.0
$MnCl_2$		20.6
ATP		1.53
NADH		0.0
NADH	NADPH (1.6 μmoles)	10.2
None	NADPH (1.6 μmoles)	53.1
None	Glutathione (8 μmoles)	49.5
None	CoASH (0.25 μmole)	25.6

Note: Each reaction mixture contained 100 μmoles of phopsphate buffer (pH 7.0), 2.5 μmoles of $MnCl_2$, 1.6 μmoles of NADH, 5 μmoles of ATP, 0.1 μmole of (1-^{14}C) octanoyl CoA (4.5×10^4 counts per min), 0.5 μmole of acetyl CoA and 1.7 mgm. of the partially purified enzyme system. The final volume was 1.0 ml; incubation was at 38°C for 1 hr.

(*From* Kanemasa and Goldman, *Biochim. Biophys. Acta* **98** (1965) 476.

TABLE 10. *Requirements for the incorporation of octanoyl CoA into long-chain fatty acids.*

Omission	Addition	Octanoyl CoA incorporated (mμmoles)
None		35.2
$MnCl_2$		19.9
$MnCl_2$	$MgCl_2$ (2.5 μmoles)	33.7
ATP		0.0
NADH		0.68
NADH	NADPH (1.6 μmoles)	8.2
None	NADPH (1.6 μmoles)	34.4
None	Glutathione (8 μmoles)	37.4
None	Acetyl CoA (0.5 μmole)	46.1

Note: Each reaction mixture contained 100 μmoles of phosphate buffer (pH 7.0), 2.5 μmoles of $MnCl_2$ 1.6 μmoles of NADH, 5 μmoles of ATP, 0.1 μmole of (1-^{14}C) octanoyl CoA (4.5×10^4 counts per min), and 1.7 mgm. of the partially purified enzyme system. The final volume was 1.0 ml; incubation was at 38°C for 1 hour.

(*From* Kanemasa and Goldman, *Biochim. Biophys. Acta* **98** (1965) 476).

TABLE 11. *Distribution of ^{14}C in fatty acids synthesized from (1-^{14}C) octanoyl CoA.*

Expt. no.	Fatty acid degraded	^{14}C in carboxyl position: Counts per min.	^{14}C in carboxyl position: % of total ^{14}C
I	C_{16}	46	51
	C_{16}[a]	59	47
	C_{18}	65	23
	C_{20}	62	26
	C_{24}	102	38
II	C_{16}	72	47
	C_{16}[a]	40	46
III	C_{16}	50	20
	C_{16}[a]	25	16
	C_{18}	85	10

Note: For Expt. I and II, the reaction mixture contained 200 μmoles of phosphate buffer (pH 7.0), 4 μmoles of $MnCl_2$, 8 μmoles of NADH, 10 μmoles of glutathione, 10 μmoles of ATP, 1.4 μmoles of ATP, 1.4 μmoles of (1-^{14}C) octanoyl CoA (3.8×10^5 counts per min) and 4.5 mgm. of the partially purified enzyme system. The final volume was 2.0 ml. Incubation was at 38°C for 3 hours.

For Expt. III, the reaction mixture was as above *except* that 3.5 μmoles of acetyl CoA was also added.

[a]Decarboxylated after rechromatography on an analytical column.

(*From* Kanemasa and Goldman, *Biochim. Biophys. Acta* **98** (1965) 476).

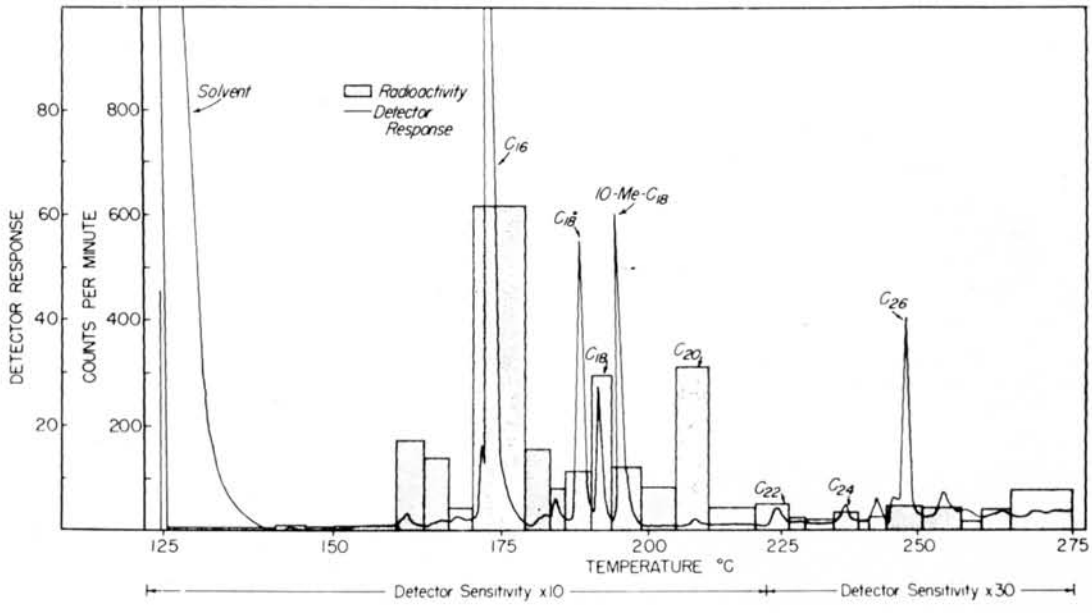

FIG. 15. Chromatography of the fatty acids synthesized from decanoyl CoA. Experimental conditions were the same as described in the legend of Figure 14 except that the substrate was (1-^{14}C)-decanoyl CoA (7.4×10^4 counts per min.) (*From* Kanemasa and Goldman, *Biochim. Biophys. Acta*, **98** (1965) 476.

complex fatty acids through this amazingly inefficient mechanism. The first clue that something different was going on came from experiments in which the specificity of the substrate was investigated([7]). The data of Table 7 show that if the medium chain fatty acid, octanoic acid, is added to the basic reaction mixture in which fatty acids are synthesized from acetate, the incorporation of acetate into fatty acids is greatly increased. This effect seems to be maximal at the chain length of 8 carbons (Table 8). The system could be simplified by the use of the acyl CoA's instead of the free fatty acids and the activating system (Table 9). There is an absolute requirement for ATP for the synthesis of long chain fatty acids from labeled octanoyl CoA and unlabeled acetyl CoA.

We then found (Table 10) that acetyl CoA was not necessary for the synthesis of the fatty acids. We were apparently following the direct synthesis of long chain fatty acids from labeled octanoyl CoA. It was of considerable importance to learn which fatty acids were being synthesized by this new system. A gas-liquid chromatographic analysis of the products of the reaction is shown in Figure 14. The substrate for this reaction was labeled octanoyl CoA. It is seen that radioactivity is incorporated strongly into the C_{16} peak and into the C_{24} peak. It is important to note that we have never in the past noted any significant incorporation into the C_{24} peak of mycobacterial fatty acids. The C_{16} peak was recovered and rechromatographed on an analytical column; the data for that run are shown in the upper insert of Figure 14. The various fatty acid fractions were recovered and decarboxylated; the ^{14}C content of the carboxyl carbon was measured. Table 11 presents the data on the recovery of the labels from the various fatty acids that were isolated from the reaction mixture by means of gas-liquid chromatography. It is evident that a new mechanism must be present to account for these results. Note that 50 per cent of the isotope content of C_{16} and 38 per cent of the isotope content of C_{24} are in the carboxyl position. The only mechanism that can give such results is one in which direct condensation of fatty acids takes place to yield new fatty acids. If this is indeed so, then in an analogous test system we should predict

that two molecules of C_{10} will condense to yield C_{20}. The data of Figure 15 show this to be the case. The substrate was labeled decanoyl CoA. The reaction products were isolated and chromatographed. For the first time in our experience significant label was recovered at the C_{20} peak; 50 per cent of the label was in the carboxyl carbon.

These results demonstrate that mycobacteria have a unique pathway for the biosynthesis of long chain fatty acids. As far as we know this condensation reaction is not present in animal cells and this is the basis for wishful thinking: a metabolic control over this reaction would probably be completely innocuous to the host, since this reaction is unknown to the host. Here is an ideal point for specific metabolic interference.

In summary; the mycobacteria have metabolic systems that in some instances are identical with those of host cells and in other instances are totally different. We have now shown two unique examples of metabolic controls over these systems and hope that through extensions of work such as reported here we will discover specific and very effective measures for blocking bacterial growth.

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DISCUSSION

Dr. Long. Thank you, Dr. Goldman. Only those who have had a chance to follow this subject of investigation for some 50 years can appreciate the degree of sophistication that has developed in modern research. Dr. Goldman has given us a remarkable picture of today's research on the mechanisms involved in oxidation and the nature of enzyme action, which I hope will prove useful for all here who go on with work on the leprosy organism itself. I will now ask Dr. Norman E. Morrison, Microbiologist in the Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory at the School of Hygiene and Public Health, Baltimore, to open the discussion of this extremely interesting and important subject.

Dr. Morrison. I would like first to congratulate Dr. Goldman on a remarkably lucid presentation concerning the difficult problem of potential control mechanisms involving mycobacterial enzymes that function in the oxidation of NADH and in the synthesis of trehalose-6-phosphate and fatty acids. It is probable that the systems under discussion are of fundamental importance for mycobacterial metabolism when cells are growing either *in vitro* or *in vivo*. This work indicated that there may well be a basis for the incorporation of AMP into medium being tested in leprosy cultivation work.

Information currently available (Kusaka *et al. J. Bact.* **87** (1964) 1383-1388) suggests that the bovine tubercle bacillus is able to expand and reorganize its energy-yielding membrane systems on isolation from the host. This poses the question if the failure of *in vitro* growth of the leprosy bacillus is due to its inability to achieve a similar change. However, while it seems unlikely that the leprosy cultivation problem can be solved simply in terms of membrane physiology, it is nevertheless important to understand the mechanisms by which the tubercle bacillus changes and then controls its energy-yielding membrane systems after transference from host to culture medium.

Dr. Goldman has presented some provocative concepts entirely new in the field of respiratory regulation. Essentially, his data have led to cumulation of the hypothesis that the rate of membrane NADH oxidation is regulated by the ADP/ATP and NADH/NAD ratios of the cell. The hypothesis proposed is that when ATP utilization occurs at a fast rate AMP levels will increase and result in an activation of the NADH oxidizing system by promoting an allosteric effect or configurational change at the catalytic binding site on the membrane particles. This results in an acceleration of NADH oxidation and increased ATP formation. The essential experimental detail in this work lies in the ability not only to demonstrate an activation effect by AMP, but also to show that the increase in NADH oxidation remains long after the activating compound, i.e., AMP, has been removed from the membrane particles. An unexpected result obtained from kinetic analysis was the finding of bireactant kinetics for NADH oxidation. This result, however, may be not unexpected, since the overall reaction involving the reduction of molecular oxygen to water requires the transport of four electrons from two molecules of NADH. Could it be then that the active center of the membrane enzyme binds not only two molecules of NADH, but also one molecule of oxygen, and that the sites are catalytically active in the simultaneous transport of four electrons to molecular oxygen? If it were not for the results obtained, demonstrating activation after AMP removal, it would probably have been assumed that the AMP effect was mediated by competing with or offsetting the NAD inhibition of the enzyme at the catalytic site.

It is noteworthy that the H37Ra particles, isolated after alumina grinding, are unable to phosphorylate. This raises two important questions, first, if the isolation procedures have been drastic enough to render considerable changes in the membrane systems coupling oxidation to phosphorylation and, second, if tightly-coupled phosphory-

lating particles, such as those obtained from *M. phlei,* would undergo AMP activation. Furthermore, it may be questioned if sufficient evidence is really available to indicate that control of respiration in bacterial systems is independent of ADP or ATP levels. The original Chance hypothesis that ADP levels control respiration has recently been expanded; it now appears that the ATP/ADP Pi quotient is a more nearly correct expression of respiratory modulation, particularly in explaining situations of reversed electron transport. While it is conceivable that "loose" or perhaps more "primitive" mechanisms couple oxidation to phosphorylation in bacteria, the results of Brodie, Gray and their co-workers show that the tightly-coupled mycobacterial phosphorylating systems can yield P/O ratios that are similar to mitochondrial systems.

It would be of interest to hear Dr. Goldman's comments on further aspects of the control of the NADH oxidizing system from H37Ra. For example, what is the effect of very high levels of AMP? Is there any inhibition? Again, what is the effect of the other half of the NAD molecule, i.e., nicotinamide mononucleotide? And finally, have sufficient concentrations of orthophosphate been tested in order to eliminate any activation by this compound? It would seem unlikely that the particles contain any AMP-splitting activity. The possibility of an orthophosphate activation effect was mentioned in view of results obtained some years ago with nonphosphorylating particles from *M. smegmatis.* These particles actively oxidize NADH and L-malate. The L-malate oxidizing system was activated by 2-6 mM orthophosphate when the reaction was run at 37°C in tris or imidazole buffers. The orthophosphate activation was independent of added magnesium ions and was not inhibited by fluoride. A similar orthophosphate activation effect has been reported for the oxidation of succinate by the Keilin-Hartree particle and the mitochondrial ETP particles.

In summary, then, consideration should be given to the possibility that highly significant differences may occur in control mechanisms operating in saprophytic as compared to pathogenic mycobacteria. Only the future can tell whether the recognition of such differences, particularly in energy-yielding mechanisms, can have successful application to the leprosy cultivation problem.

Dr. Goldman. I am afraid that an answer may call for an entire new presentation, which I would prefer not to make. Very briefly, however, are the particles uncoupled by the mode of cell disruption? I do not know. We have never been able to get oxidative phosphorylation regardless of the method of breaking the cells. We have not been able to make spheroplasts, which is the obvious way to try it. It is always possible that they have been uncoupled by the mode of breaking. One plug here on the question of *M. phlei* and *M. tuberculosis.* These are not the same organisms, and, Dr. Gray, Dr. Brodie and other friends to the contrary, they are so totally different that they cannot be considered in the same discussion. I have yet to see any real difference if I look at slides of the cytochrome system of *M. phlei,* yeast, and *Azotobacter vinelandii.* On quick inspection I have yet to be able to identify the organism from which the cytochrome comes. The fact remains that the tightly coupled phosphorylation systems are unique to the rapid growers in the bacterial species. There is no inhibition by high concentrations of NAD. We have taken this reasonably high—to perhaps 10 or 20 times the maximal concentration needed—and get no inhibition. This is highly specific for NAD. Inorganic phosphate has no effect on the system—in fact it is not present in it. If you add it, it makes no difference. Inorganic phosphate is the obvious compound to look for when you are dealing with oxidative phosphorylation. It just does not go in. There is no AMPase, or ATPase in the particles. If we put in C^{14}-labeled AMP, we recover it quantitatively following the reaction. We have tried a number of different ways to demonstrate oxidative phosphorylation. We have attempted to couple electron transfer to ion transport, and have tried to couple

it to polyphosphate formation—all unsuccessfully. I wish I knew a reason for it, but I do not.

Dr. Long. Dr. Goldman, may I ask one question that may be in the minds of all of us here? You have mentioned important differences between a typical saprophyte, *M. phlei*, and a pathogenic organism, the tubercle bacillus. How constant do you find their enzyme activities for a given strain. Do you find differences from time to time?

Dr. Goldman. With our H37Ra laboratory strain, Dr. Long, we find essentially no differences except for those depending upon the age of the organism. The effect of allosteric control on the cytochrome respiratory system, which I discussed first, is maximal when the organisms are about two weeks old. If you go to much older organisms, four weeks old on surface culture, the effect essentially disappears. However, recent work in our laboratory, which is going on at the moment, indicates that even the particles from the old bacteria can yield a soluble enzyme that shows allosteric inhibition or activation, even though the particles themselves do not; so there is a change in the membrane physiology of the organism dependent upon age. But if you hold age constant, there is remarkably little variability.

Dr. Long. Thank you. I am sure that the point is of importance for those who are attempting to grow *M. leprae*, because, as we see the organisms in lesions, there is an enormous variation in age, and enzyme variation must be important in metabolic processes in life.

Dr. Hanks. This question of oxidative phosphorylation is a fascinating one. Everyone believes that when H37Rv and H37Ra grow they conduct oxidative phosphorylation. I think in one of the slides you had one of the possible clues. Pellicle-grown cells, as Dr. Tepper has recently been learning, are so far from homogeneous that they are handicapped by the time the crop has been produced. If you take the saprophytes and you wish cells capable of giving good oxidative phosphorylation when ruptured, the first consideration is to grow them at maximal rates on a shaker. The second point is that it is possible to learn, in the case of organisms performing poorly in oxidative phosphorylation, whether the defect may be in the particles or possibly in the low content of soluble cofactors. I am sure you will recall the experiments of Brodie and Gray in which they took particles, for example from *Azotobacter,* which would perform immeasurably better when supplemented with the supernatant factors from *M. phlei* than when given the supernatant fractions from themselves. These cross-switches of particles from more competent supernates from H37Ra, to supplement the less competent particles of the H37Ra, might help us to learn in which of the components the more serious limitations reside.

Dr. Goldman. I prefer not to go into the problem of homogeneous or nonhomogeneous populations, except to recognize that they are obviously there. However, we are able to reproduce our experiments from preparation to preparation. We are currently attempting to obtain sufficient cells in a consistent phase of growth to avoid this problem. We have obviously tried the addition of supernatant factors to the particles, and this has been totally unsuccessful, whether we keep them in the dark, expose them to the light, heat them, or keep them plain. The effort has been uniformly unsuccessful—I do not know the reason. I am sure that these particles phosphorylate, but we cannot prove it.

Dr. Segal. I would like to ask a question on each section of your talk. First of all, I missed your response to Dr. Morrison's question as to whether you think an electron reversal effect is involved in your NADH oxidase study. Second, in relation to the studies of an inhibitor, I am curious to know if you have given any thought to the relationship of the phenomenon to the Jacob and Monod repressor-operon model. Third, in terms of your fatty acid synthesizing system, I cannot recall all the details of Dr. Konrad Bloch's classification system of microorganisms, plants and animals in rela-

tion to fatty acid synthesis, and I wonder where your findings would fit in that scheme.

Dr. Goldman. I will take these in reverse order because I did not put the last question down. The extracts we deal with do not synthesize unsaturated fatty acids. They synthesize only normal saturated fatty acids. If you let them run on malonyl CoA, they will go up to C_{36}; we have had tracings that indicate that even C_{40} is present. They simply go, but they do not form any unsaturates, and they do not form any branched chain fatty acids in their present form. We have no information on repression. The activities that we have been dealing with have been directly on the isolated enzyme and have nothing to do with the enzyme-forming system. Third, we have no information showing electron reversal, using the cytochrome chain of, let us say, succinate to NAD.

Dr. Morrison. I wonder, Dr. Goldman, if Mycoribnin has any growth inhibitory effects, and second, if avidin sensitivity plays any part.

Dr. Goldman. We have not tested this inhibitor to see if it has growth inhibitory activity in the intact cells, but, on another point, which is similar, we have found that if we add NAD in moderately high concentrations to the growing cells it will inhibit growth. This result might be related to the allosteric effect—we do not know—but Mycoribnin as such has no inhibitory effect. I do not think that the bicarbonate effect has anything to do with biotin or avidin. We have no evidence of that whatsoever. It takes place at extremely low concentrations. The only time we found avidin effects was in the fatty acid-synthesizing system from acetyl CoA with bicarbonate as a requirement.

Dr. Pappagianis. I would like to ask if growth of strain H37Ra in the presence of bicarbonate or an elevated pH imbues the strain with any different properties of pathogenesis, i.e., if any gain in virulence is acquired in the accompanying triglyceride synthesis.

Dr. Goldman. I have no information on that. I suspect that Dr. Guy Youmans could tell us if he were here.

Dr. Long. We will write to him. This is an informal conference, and we do not quit when we leave here. Our object is to induce those who are interested to talk and write to each other. I think, Dr. Pappagianis, that you might ask that question profitably of Dr. Youmans.

Dr. Pappagianis. May I ask one more question? H37Ra is notorious because of its performance in animals. When you put it into the animal there is no deficiency of bicarbonate or CO_2.

Dr. Middlebrook. We culture our mycobacteria routinely under 2-5 per cent CO_2. Up to 10 per cent we find no change in the morphology of H37Ra with increases in concentration of CO_2, and subcultures behave in animals just as the parent strains do when cultivated under lower concentrations of CO_2. I think there might be something in this problem, because recent experiments on aerogenic infection of guinea-pigs with H37Ra show it to be a pretty good infecting agent for this animal. It multiplies very well—I would say as well as $H37R^s$—in the lungs of guinea-pigs for the first two weeks of the infection. It is after that time that it becomes embarrassed. This is the opposite of what one might expect, if increase in CO_2 were involved in increase in virulence—don't you think?—because the CO_2 concentration in inflammatory lesions should be increased. Many other events take place, of course, in inflammatory lesions, but I think it is interesting that the organisms do grow beautifully at first. It is only a result of the development of some sort of host responsiveness that H37Ra shows behavior different from that of $H37R^s$ in guinea-pigs.

Dr. Reich. In response to a question about the differences in biochemical activity between *M. phlei* and *M. tuberculosis*, Dr. Goldman stated that he would like to stress the fact that these are two different organisms. I would like to ask Dr. Goldman, then, what hope he can hold out for

us that the biochemical findings of any of the mycobacteria that can be cultivated can lead us to the cultivation of *M. leprae*.

Dr. Goldman. There is no answer to it, Dr. Reich. I am sorry. Every organism has had its difficulties as far as the laboratory people are concerned in reproducing its growth. I have nothing to do with growing the organism. The Proskauer and Beck medium we are using was developed about 1896. It works beautifully. I do not know if any of the growth and nutritional studies will reflect back on *M. leprae*. What we are concerned with is the metabolic activities of this organism after it has been grown.

Dr. Long. I hope it will all apply to our study of *M. leprae*. We shall have to close the discussion at this point.

The next speaker is Dr. R. E. Kallio, Professor of Microbiology in the College of Medicine of the University of Iowa. Dr. Kallio is well known for his investigations of several phases of mycobacterial metabolism,. including the utilization of hydrocarbons as well as lipids, a subject that has been mentioned several times already this morning. Dr. Kallio will speak on the "Physiologic implications of hydrocarbons and lipids in mycobacteria and related forms."