INCORPORATION OF AMINO ACIDS INTO THE PROTEINS OF *Micrococcus lysodeikticus*

Thesis by

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ABSTRACT

Lysis of Micrococcus lysodeikticus cells with lysozyme in the presence of high concentrations of sucrose has yielded a particulate system, distinct from intact cells, that carries out the incorporation of amino acids into protein. All seven amino acids which have been tested have been incorporated.

The system was sedimentable, had a high endogenous respiration, and required the continued presence of high sucrose or KCl concentrations for activity. The importance of oxidative reactions for incorporation was indicated since anaerobiosis and 2,4-dinitrophenol treatment inhibited incorporation. Ribonuclease treatment diminished incorporation; desoxyribonuclease treatment accelerated incorporation. The activity of the particulate system was inversely related to the concentration at which these particles were assayed. Stimulation of incorporation was observed upon addition of unlabeled amino acids implying a net synthesis of protein molecules under these conditions. From data obtained on the composition of the free amino acid pool and the rates of amino acid incorporation it was concluded that in the absence of added amino acids, amino acid incorporation occurred either via an exchange reaction or that it reflected synthesis of new protein molecules with concomitant breakdown of pre-existing protein to amino acids.

It was shown that radioactive protein isolated after incubation with radioactive leucine, contained leucine as its sole radioactive constituent. The conversion of leucine to α-ketoisocaproic acid was also shown.

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I. INTRODUCTION

Ever since knowledge accumulated concerning the chemical nature of proteins and their functions in living things, the problem of protein biosynthesis has been of major interest to biochemists. The early work in this field was limited to the grosser aspects of protein metabolism such as the nitrogen balance phenomenon and protein catabolism. The scanty progress toward understanding the chemical steps in the synthesis of a protein was due in part to difficulties inherent in studying a very slow process and in part to the lack of basic biochemical techniques both for the handling of tissues and the identification and estimation of small amounts of substances. Certainly the greatest technical innovation was the use of isotopically labeled metabolites; its first application in the hands of Schoenheimer (1) verified and expanded the earlier observations of Borsook and Keighley (2) and effectively demolished the theories of Folin (3) which had held sway for 30 years (See Part I, B). The problem of protein synthesis was now open to attack, and a number of topics related to the problem have been extensively explored in recent years, among them the synthesis of small peptides and quasipeptides, incorporation into protein of labeled amino acids in vivo and in vitro, the role of nucleic acids; these are discussed below.

A. Formation of Peptide Bonds in Small Molecules

The enzyme catalyzed formation of peptidic bonds in small molecules has been extensively studied for its own sake and for its possible

significance in understanding protein peptide bond formation.

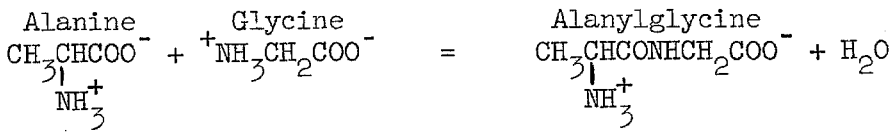
1. Thermodynamic Considerations

In Table 1 are listed some thermodynamic quantities for the formation of several small peptides. For dipeptides in equilibrium with amino acids, the position of the equilibrium is far to the side of hydrolysis. For example, in the case of alanylglycine, formation of the dipeptide by mass action alone yields only $4 \times 10^{-3}\%$ synthesis when the initial alanine and glycine concentrations are 0.1M. Even when the equilibrium constant is relatively more favorable, as in the case of N-benzoyltyrosylglycinamide formation, only 5% synthesis is obtained when the initial concentration of reactants is 0.1M; at lower and probably more physiological concentrations the amount of synthesis at equilibrium is 100 times less. Table 1 also shows that the free energy of formation of a peptide is dependent on the charge distribution of the reacting moieties. The trend observed is that the more removed other charged groups are from the reacting carboxyl and amino groups, the more energetically favored is peptide synthesis. For example, two extremes may be considered (equations 1 and 2): the formation of alanylglycine and the formation of N-benzoyltyrosylglycinamide. In the former case where the reactants are both zwitterions, alanine and glycine, the ΔF of formation is 4130 calories. In the latter case only the reacting carboxyl group of the N-benzoyltyrosine and the amino group of the glycinamide are charged; here the ΔF of formation is 361 calories. This difference in ΔF's of formation leads to almost a 500-fold difference in the equilibrium constants.

Table 1. Free Energy of Formation of Some Small Peptides, Equilibrium Constant and Degree of Synthesis by Mass Action at Different Initial Concentrations of Reactants

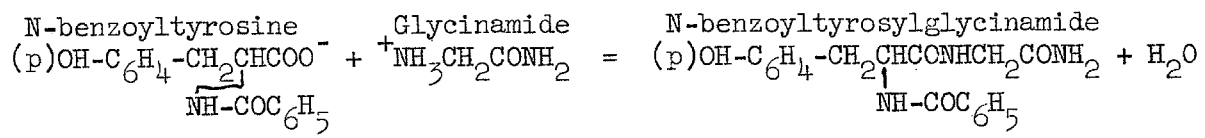
Reaction	ΔF Cal.	Equilibrium Constant K	Per Cent Synthesis at Equilibrium at Initial Concentrations of Reactants 0.1M	.01M	.001M	Reference
DL-alanine + glycine = DL-alanylglycine + H_2O	4130 at 37.5°	.00125	4×10^{-3}	$\approx 10^{-4}$	$\approx 10^{-5}$	(5, 6)
2 glycine = glycylglycine + H_2O	3590 at 37.5°	.00299	3.3×10^{-2}	3.4×10^{-3}	$< 10^{-3}$	(5, 6)
L-aspartate + NH_4^+ = L-asparagine + H_2O	3460 at 37.5°	.00367	4×10^{-2}	4×10^{-3}	$< 10^{-3}$	(7)
DL-leucine + glycine = DL-leucylglycine + H_2O	3315 at 37.5°	.00467	4.2×10^{-2}	5.3×10^{-3}	$< 10^{-3}$	(5, 6)
Benzoate + glycine = hippurate + H_2O	2630 at 37.5°	.0142	1.4×10^{-1}	1.4×10^{-2}	1×10^{-3}	(5, 6)
Benzoate + glycylglycine = benzoylglycylglycine + H_2O	1100 at 25°	.1564	1.5	1.6×10^{-1}	9.5×10^{-3}	(5, 4)
N-benzoyltyrosine + glycine-amide = N-benzoyltyrosyl-glycineamide + H_2O	361 at 37.5°	.5582	5.0	5.5×10^{-1}	5.8×10^{-2}	(8)

1.



$\Delta F = 4130$ cal.

2.



$\Delta F = 361$ cal.

It may also be seen that if the nonreacting charged groups are further removed than they are in an amino acid, as in a peptide, the free energy change in formation is less (cf. benzoylglycine and benzoylglycylglycine formation).

With these facts in mind it would be difficult to view protein synthesis as a mass action reversal of hydrolysis without assuming special conditions leading to a driving of the reaction towards synthesis. These could be product removal or high amino acid concentrations at the site of synthesis. In any view, however, a coupling with energy donating reactions would be required either in maintaining these special conditions or in forming more reactive intermediates.

2. Protease Catalyzed Synthesis and Transfer Reactions

The synthetic reactions catalyzed by proteases are of several types. When the proper substrates are chosen, considerable synthesis may occur

because of either a favorable equilibrium constant or because of sparing solubility of the product formed. For example, papain readily catalyzes the formation of hippurylanilide starting from hippuric acid and aniline; here the ΔF of formation is about -5000 calories as calculated from equilibrium data (9), and the equilibrium actually favors synthesis, quite unlike the usual case of peptide formation. Bergmann and his collaborators have described many synthetic reactions which are promoted by formation of slightly soluble products (10). N-benzoyltyrosylglycinanilide in yields as high as 65 per cent have been observed to crystallize from solution in the chymotrypsin catalyzed reaction of N-benzoyltyrosine and glycinanilide.

In addition to the kinds of reactions mentioned, proteases have been shown to catalyze group transfer reactions, transamidations and transpeptidations, during the course of hydrolytic cleavage of various peptides, amides, and esters. The reaction course is outlined in equation 3.

3.

$$RCO\text{-}R_1 + NH_2\text{-}R_2 \xrightarrow{\text{Enzyme}} RCO\text{-}NHR_2 + R_1H \xrightarrow[H_2O]{\text{Enzyme}} RCOOH + NH_2R_2 + R_1H$$

R_1 = $-NH_2$, $-OR$, amino acid derivative

R_2 = $-H$, $-OH$, amino acid or derivative

Fruton and co-workers have found that in the presence of glycinamide the hydrolysis of the amide bond of benzoyl-tyrosinamide by chymotrypsin was accompanied by intermediate formation of benzoyltyrosylglycinamide (12).

4.

N-benzoyltyrosinamide + glycinamide = N-benzoyltyrosylglycinamide + NH_3 $\xrightarrow{H_2O}$ N-benzoyltyrosine + glycinamide + NH_3

In this transamidation reaction 14 per cent replacement was observed when the benzoyltyrosinamide was 40 per cent hydrolyzed. An example of a transpeptidation reaction similar in nature has been described by Johnston et al. (13). By the use of N^{15} labeled glycinamide, it was shown that exchange of the glycinamide moiety of benzoyltyrosylglycinamide (BTGA) with free glycinamide had occurred during hydrolysis of BTGA with chymotrypsin (equation 5).

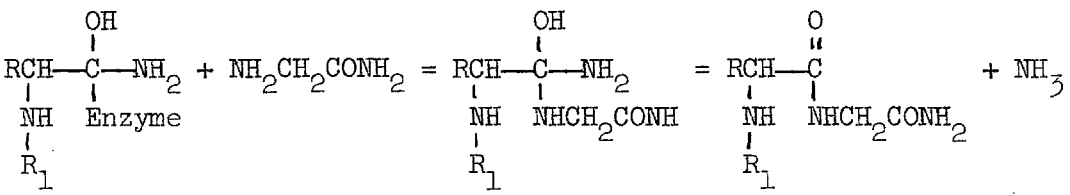
5.

N-benzoyltyrosylglycinamide + glycinamide(N^{15}) = N-benzoyltyrosylglycinamide(N^{15}) = N-benzoyltyrosine + glycinamide(N^{15})

Replacement to the extent of 17 per cent had occurred after 42 per cent hydrolysis of BTGA. It should be emphasized that these reactions eventually proceed to almost complete hydrolysis and that a new peptide bond is made at the expense of a pre-existing peptide or amide bond.

Fruton has postulated a transient enzyme-substrate intermediate to account for these protease catalyzed reactions (13). In the case described in equation 4, this would be

6.

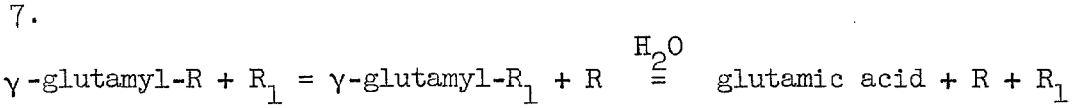


(R = (p)OH-C_6H_4-CH_2-; R_1 = COC_6H_5)

An acyl-enzyme complex has also been envisaged as a possible intermediate (4). The further reaction of the activated complex with various reagents is, under either view, then thought to govern the course of the reaction; reaction with water would yield hydrolysis, and reaction with replacement agents such as amino acid derivatives, ammonia, or hydroxylamine would lead to formation of peptides, amides, or hydroxamic acids, respectively. This conservation of "bond energy" in an enzyme-substrate complex is similar to mechanisms postulated for polysaccharide reactions (14).

Other examples of transpeptidation reactions were described by Hanes et al. (15). They demonstrated that crude kidney extracts would catalyze the following reactions:

7.



Where R could be cysteinylglycine, glutamic acid, tyrosine or phenylalanine and R_1 could be almost any amino acid or peptide.

A crude enzyme system was also described which catalyzed the following reaction:

8.

$$\text{Glycyl-R} + R_1 = \text{glycyl-}R_1 + R \overset{H_2O}{\leftrightharpoons} \text{glycine} + R_1 + R$$

Where R was glycine, tryptophan, leucine, phenylalanine, or glycylglycine; R_1, the acceptor group, could be any amino acid.

With these enzymes the reactions went to complete hydrolysis in time. It was considered by the authors that γ-glutamyl activation of amino acids may play a part in protein synthesis by using the energy of peptide bonds already formed in exergonic reactions. Besides being difficult to see how any other than γ-glutamyl bonds are formed, their proposal was rendered unlikely by the work of Hendler and Greenberg (16). In several different systems these authors showed no stimulation of amino acid incorporation into protein by γ-glutamyl compounds. Also free glycine incorporation was more efficient than glycine added as γ-glutamyl glycine. In summary, there exists no convincing evidence for the role of proteases or specific transpeptidases in protein synthesis.

3. Peptide Syntheses Coupled with Exergonic Reactions

The formation of hippuric acid from benzoic acid and glycine by mass action is negligible (Table 1), hence for any significant synthesis a coupling with an exergonic reaction would be required. It was shown early that synthesis of hippuric acid in in vitro systems was linked to oxidative processes (17). The mechanism of the reaction was studied by Chantrenne (18) and later by Schachter and Taggart (19, 20). The reaction sequence was proved to be:

As indicated, synthesis occurs with the stoichiometric breakdown of ATP to yield ADP and orthophosphate. However, only catalytic amounts of ATP, or better ADP, and phosphate or arsenate are required for the transferase reaction. Synthetase activity is increased in the presence of cysteine; both the synthetic and transfer reactions are inhibited in the presence of p-chloromercuribenzoate, implying a free -SH group requirement. Since there was no indication of free reactive intermediates in the reaction, attention was turned to the possibility of enzyme bound intermediates. The formation of either an enzyme-phosphate or an enzyme-ADP complex giving rise to an enzyme-glutamate complex (which could then react with ammonia) has been excluded. This mechanism would require equilibrium of labeled phosphate with ATP in the presence of glutamate. This does not occur (25). Boyer et al. have shown that when the reaction is carried out using O^{18} in the γ-carboxyl of glutamic acid, the labeled atom is found without dilution in the organic phosphate formed (26). This result implied acyl-phospho anhydride formation at some step in the reaction. A scheme which accounts for the data, involving an enzyme-bound form of γ-glutamyl-phosphate, is represented in equation 12.

12.

a) $\text{Enzyme} + \text{ATP} + \text{glutamate} = \text{enzyme}\begin{matrix}\nearrow \text{ATP} \\ \searrow \text{glutamate}\end{matrix}$

b) $\text{Enzyme}\begin{matrix}\nearrow \text{ATP} \\ \searrow \text{glutamate}\end{matrix} = \text{enzyme}\begin{matrix}\nearrow \text{ADP} \\ \searrow \gamma\text{-glutamyl-phosphate}\end{matrix}$

c) $\text{Enzyme}\begin{matrix}\nearrow \text{ADP} \\ \searrow \gamma\text{-glutamyl-phosphate}\end{matrix} + NH_3 = \text{glutamine} + \text{ADP} + H_3PO_4 + \text{enzyme}$

The binding of the reactants could occur through the mediation of the metal ions required for the overall reactions. γ-Glutamyl-phosphate has been shown to react non-enzymatically with ammonia to give glutamine and with hydroxylamine to form hydroxamic acid, both reactions proceeding to complete synthesis (27).

Two enzymatic steps have been distinguished in the biosynthesis of glutathione.

13.

a) Glutamic acid + cysteine + ATP = γ-glutamylcysteine + ADP + H_3PO_4

b) γ-Glutamylcysteine + glycine + ATP = glutathione + ADP + H_3PO_4

Magnesium and potassium ions are the only requirements for the synthesis apart from those described in equation 13. As was the case for glutamine synthesis, no free intermediates have been found. The reaction mechanism as far as is known is the same for both steps in the synthesis.

14.

a) Enzyme + ATP = enzyme-P + ADP

b) Enzyme-P + glutamic acid (γ-glutamylcysteine) =
enzyme-glutamic acid (glutamyl cysteine) + P

c) Enzyme-glutamic acid (glutamyl cysteine) + cysteine (glycine) =
enzyme + glutamyl cysteine (glutathione)

Evidence for the first step (equation 14a) was obtained by demonstrating that ATP became labeled when ADP^{32} and ATP were incubated with the enzymes (29, 30). When glutamate, ATP and labeled phosphate (P^{32}) were incubated with the enzyme, the ATP became labeled; this labeling diminished when cysteine was also added to the system. This provided evidence for the last two steps (equations 14b and 14c).

In the reactions thus far discussed, carboxyl group activation has always been a prelude to the synthesis of a new peptide bond. In the case of protease catalyzed reactions, the new bond is formed at the expense of a pre-existing bond. In the synthesis of hippuric acid, glutamine or glutathione, the peptide bonds are formed at the expense of pyrophosphate bond cleavage in the ATP molecule. It seems very likely (although no direct proof is yet available) that carboxyl group activation could play a key role in protein synthesis; indeed, if ATP turns out to be the direct energy donor for amino acid activation, one of the reaction mechanisms discussed above may be a faithful copy of the initial steps of protein synthesis.

B. Incorporation of Amino Acids into Proteins

Only that amino acid incorporation work will be discussed which has a direct bearing on the subject of this thesis and on the problem of mechanism. A more detailed discussion of this subject will be found in recent reviews (31, 32).

1. The Dynamic State

A theory of protein metabolism proposed by Folin and considered for many years as valid, held that the nitrogen excretion in an animal was almost completely due to the metabolism of exogenous protein. Only a small amount of this nitrogen excretion was thought to be derived from the pre-existing proteins of the animals in the course of a "wear and tear" process called "endogenous" protein metabolism. This view was in part derived from the notion of treating biological systems as if they were mechanical systems. Labeling experiments have been crucial in disposing of this theory. These experiments (1,2) show that the contribution of "endogenous" metabolism to the nitrogen excretion is very high. In rats and man in nitrogen balance as much as 50 per cent of the nitrogen excretion can be attributed to the breakdown of pre-existing tissue protein. A concomitant rebuilding of tissue proteins must occur to maintain the animal in a steady state.

The rate of replacement of proteins in an animal is roughly in the range of 1 to 5 per cent per day for the whole animal. This datum was obtained by following the fate of labeled amino acids after injection. The rate of replacement varies with the animal and with the tissue studied, plasma and visceral proteins making the largest contribution (31). These findings in themselves say little about how much of the turnover observed is due to specifically intracellular breakdown and resynthesis of protein. Certainly growth of new cells and secretory phenomena, such as plasma protein formation, make a contribution to this turnover. This point has been raised recently by Hogness, Cohn, and

Monod (33). These authors found that in growing E. coli the molecules of the induced enzyme, β-galactosidase, are essentially stable once synthesized. This result has been verified by Rotman and Spiegelman (34) who also found that less than one per cent of the carbon of β-galactosidase came from cellular carbon present at the start of induction. Hogness et al. generalized their result as follows:

> " . . . there seems to be at present no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover our experiments have shown that the proteins of E. coli are static. Therefore, it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a 'dynamic state'."

It must be admitted that for animals completely conclusive evidence either way does not exist on this point. However, in the case of yeast, it is well known that induced enzyme formation can occur at the expense of an endogenous nitrogen source (35). Even in E. coli other induced enzymes such as lysine decarboxylase and a glucose utilization system do not show the stability of β-galactosidase; these data were based only enzymatic activity (36, 37) and hence it could be argued that no protein breakdown was involved. Podolsky has shown that in E. coli release of protein arginine may be as high as 8 per cent in a 30 hour period (38). It has been pointed out that the rate of turnover observed in animals would be just below the limit of detection in the experiments of Hogness et al. and Rotman and Spiegelman (34); the synthesis of protein in E. coli during growth is 1000 times faster than this turnover rate.

In animals some evidence does exist concerning the intracellular breakdown of protein molecules. When a rat is fed on a protein-poor

diet, the ribonucleic acid and protein content of the liver decreases markedly, whereas the amount of desoxyribonucleic acid remains constant, indicating a constancy in cell number (39, 40, 41). Resumption of a complete diet brings the composition back to "normal". There is a likelihood that this holds true for other tissues too. Here then is a case where a remarkable intracellular lability occurs, proteins breaking down and rebuilding under dietary stress. In this sense a very dynamic state does indeed exist.

2. Properties of the Incorporation Reaction

The incorporation of labeled amino acids into proteins has been studied in a variety of systems. These systems run the gamut of organization from intact animals to sub-cellular particles. Only one case of amino acid incorporation in a truly soluble system has been described which also seems to be unique in that only lysine is incorporated, and at that through its ε-amino group (42). The general experimental design has been to incubate some tissue with a labeled amino acid and then to isolate the protein in a state which is uncontaminated with free labeled amino acid. Although in some cases a single protein has been studied, usually incorporation has been measured in the total acid insoluble fraction of the tissue. Some of the characteristics of incorporation in these systems will be described.

a. Rate of Incorporation. Although the measured rate of incorporation of an amino acid depends on factors such as dilution by pre-existing amino acid pool, initial amino acid concentration, kind of tissue and other factors to be discussed, it can be said that the

rate is of the order of magnitude of one micromole of amino acid incorporated per gram of tissue protein per hour. Amino acid incorporation is a very general reaction concerning all tissues and all amino acids tested (31).

b. Energy Requirements. A number of experiments have indicated the requirement for some kind of energy source to drive the incorporation reaction. For example, the activity of dialyzed rat liver homogenates was restored by addition of ATP, magnesium chloride and an amino acid mixture; AMP, ADP, ATP, or α-glycerophosphate were equally effective (43). Siekevitz, working with rat liver particles, showed that for incorporation a complete system was composed of microsomes, mitochondria, ATP, $MgCl_2$ and an oxidizable substrate such as α-ketoglutaric acid (44). In an extension of this work, Zamecnik and Keller have shown that the mitochondrial fraction may be replaced by a soluble protein fraction and an ATP generating system (45). When the soluble fraction was treated to remove nucleotides, it was shown that guanosine diphosphate (GDP) stimulated incorporation although only in the presence of ATP (46). In addition, inhibition of incorporation has been observed by the addition of compounds and treatments known to interfere with production of energy rich compounds. Inhibition of incorporation has been noted with anaerobiosis (47), 2,4-dinitrophenol (44, 48, 49), malonate (43), azide (47, 49) and fluoride (49). How these energy rich compounds participate in the incorporation reaction is not yet clear. However, Hoagland has shown that the soluble protein fraction required for amino acid incorporation in the liver microsome system (45)

can catalyze the formation of amino acid hydroxamates with the concomitant hydrolysis of ATP (50). Whether this represents trapping of an amino acid derivative activated prior to its incorporation is not yet understood.

c. Organization. For incorporation to occur in the systems studied, some degree of organization seems to be required. For example, the incorporating activity of rabbit reticulocytes was completely destroyed with cell lysis (49). It has been found that liver homogenates have much less activity than slices, the method of homogenization being very critical (45, 51). The activity of these homogenates and of the particulate systems derived from them fell off rapidly with time; several hours at 0° C. resulted in loss of activity (45). Desoxycholate treatment of the liver microsome system destroyed activity (45). Similar observations have been made with bacterial systems; prolonged ultrasonic treatment led to losses in activity (52). Of course, it may not be that organization per se is a requirement for the reaction. Incorporation may indeed be demonstrated in simpler systems when a fuller understanding of the reaction is achieved.

3. Mechanism of Amino Acid Incorporation - Intermediates - Nucleic Acid Function

Of foremost interest are the nature of the reactions leading to peptide bond formation and to the determination of amino acid sequence. Attention has been turned to the importance of nucleic acids in determining protein specificity. The recognition of genetic control of enzyme formation and the direct demonstration of DNA operating in the transforming principles and in phage reproduction have been compelling

evidence in this regard. Furthermore, much indirect evidence has been obtained for the importance of RNA in protein synthesis (53, 54). Tissues (or fractions thereof) containing large amounts of RNA have rapid rates of amino acid incorporation (44, 45, 55). Somewhat more direct evidence has been the demonstration of ribonuclease inhibition of amino acid incorporation in a number of systems: cell free preparations of Staph. aureus (56) and Micrococcus lysodeikticus (57, 58); onion root tips (59); liver particles (45, 60). In short term experiments, the nucleus does not seem to be involved in amino acid incorporation. Brachet and Chantrenne showed that enucleation of Acetabularia mediterranea had no effect on incorporation for about two weeks; after that time the rate of incorporation fell off faster in the non-nucleated portion than in the one containing a nucleus (61).

Gale and Folkes have shown that depletion of nucleic acid in disrupted cells of Staph. aureus resulted in a decrease in amino acid incorporation; this incorporation was restored by the addition of staphylococcal nucleic acid preparations, DNA being somewhat more effective than RNA (62). The restorative powers of the RNA do not necessarily seem to be a specific macromolecular property since these workers have recently reported that RNA may be replaced by small amounts of di- and tri-nucleotides which stimulated the incorporation of only certain amino acids (63). It is not yet clear whether the DNA stimulation is of a similar nature. Although these experiments provide fairly direct evidence for the implication of nucleic acids in amino acid incorporation and protein synthesis (64), their precise role is not clear.

A number of workers have observed that amino acids may be incorporated into protein in a somewhat independent fashion. It has been shown that certain antibiotics and amino acid analogues would specifically inhibit the incorporation of one amino acid (65, 66). These results have been interpreted as evidence for an exchange reaction, a labeled amino acid exchanging with a non-labeled amino acid already in a preformed protein. Other evidence in this direction has been supplied by the work of Gale and Folkes who have demonstrated a reversibility in the incorporation reaction in a disrupted cell system. It was shown that when a preparation containing protein labeled with radioactive glutamic acid was incubated with non-radioactive glutamic acid, the protein became less radioactive. An energy source (ATP and hexosediphosphate) was required for both the incorporation and "excorporation" reactions (62). In the incorporation experiment, no further increase in labeling was observed when about 5 per cent of the glutamic acid residues became labeled. These authors felt that incorporation which proceeded in the absence of exogenous amino acids (i.e., no net protein synthesis) occurred only in the nucleoprotein fraction. In the studies cited in this paragraph where exchange reactions have been invoked, only acid precipitable mixtures of proteins have been studied, and, therefore, one cannot say whether a well-defined preformed protein truly enters into the reaction. There is a possibility that all such phenomena might be explained by considering that the "protein" associated with RNA actually represents intermediate stages in the synthesis of functionally important proteins. In this state the protein material could enter into exchange reactions with added amino acids. This material would

probably be acid insoluble and therefore be measured as protein in an incorporation experiment. This view is consistent with the magnitude of incorporation measured, there being enough nucleic acid present in the systems studied to match the amount of incorporation (synthesis) observed. This kind of scheme (67) has been envisaged to explain, among other phenomena, the results of Anfinsen and co-workers. These authors studied the synthesis of ovalbumin (68, 69, 70), ribonuclease and insulin (71). Tissue slices were incubated with various radioactive amino acids and then the protein in question was isolated in a homogeneous form. They demonstrated that the same kind of amino acid residue derived from different portions of the protein molecule had different specific radioactivities. The magnitude of these differences varied in a roughly direct manner with the extent of labeling, i.e., with the amount of protein synthesized. These results were interpreted as ruling out the possibility of protein synthesis proceeding via simultaneous assembly of amino acids since this view would require equal labeling. Among several possible explanations Anfinsen et al. favored one involving intermediate peptide formation (69). In the case of insulin the small amount of synthesis (ca. 1γ/10 g. pancreas/4 hours) would be consistent with a small unlabeled pool of intermediates possibly associated with RNA. In this view the slowness of the reaction may turn out to be a hidden blessing. The exchange phenomena may thus be viewed as reflecting an integral aspect of protein synthesis (No originality is claimed for any of these ideas).

Allfrey et al. (60) analyzed the microsomal nucleoprotein from liver and pancreas and concluded that the protein component was not

especially rich in the basic amino acids. These authors concluded that the possibility of this protein representing precursor material was not inconsistent with their studies on the course of incorporation in various cell fractions. Similar results had been obtained by Siekevitz (44).

II. STATEMENT OF THE PROBLEM

It was thought that a useful approach to the problem of protein biogenesis would be the development of a cell-free system with which the incorporation of amino acids into proteins could be studied. Since protein synthesis is a slow reaction, it was considered profitable to use an organism with a high metabolic rate, namely a bacterium. At the time this investigation was started, the only comparable systems investigated at all were particulate systems from liver. Since that time a similar bacterial system has been described by Gale and Folkes (referred to in Part I). A preliminary account of the early portion of the present investigation has been presented (57).

Cell-free material possessing incorporating activity had been obtained at an early stage of this investigation. However, it was thought premature to attempt experiments designed to answer questions of mechanism until some basic knowledge could be obtained concerning the preparation and assay of the system. Indeed, many experiments failed because some of the variables to be discussed were not understood.

III. MATERIALS AND METHODS

A. Description of Organism and Its Culture

The organism used in these studies was Micrococcus lysodeikticus (American Type Culture Collection No. 4698), a strictly aerobic, gram-positive micrococcus (72). A particular advantage of this organism is that its cell wall material is specifically depolymerized by lysozyme, an enzyme found ubiquitously in nature (73). Lysozyme has been crystallized from egg white, a rich source of the enzyme.

No chemically defined growth medium has been described for this organism (72, 74). A solid growth medium was used in this study since growth on a liquid medium led to erratic results. The medium was 90 g. Difco Bacto nutrient agar, 3 g. Difco Bacto yeast extract, 15 g. glucose, 300 ml. tap water and 2700 ml. distilled water. About 600 ml. of this medium was sufficient to cover the bottom of a rectangular enamel tray (8" X 14" X 2 1/2"). These trays were covered with heavy wrapping paper. The organism was transferred about once a month on the medium described and slants were stored at 10° C.

The inoculum was prepared as follows. A sub-culture slant was incubated for a day at 35° C. From this sub-culture slant new slants were prepared equal in number to the trays to be subsequently inoculated. These new slants were incubated for one day at 35° C., and the organisms were eluted from the agar of these slants by shaking with several milliliters of 1% sodium chloride solution. These suspensions were combined and diluted with enough saline to provide an 8 ml. inoculum for each tray. This inoculum was distributed uniformly over the agar

surface with a glass spreader. An inoculum of less than 8 ml. led to incomplete spreading; a larger volume also resulted in diminished growth. The trays were then incubated for 20 hours at 35° C. Only the operations up to this point were performed with sterile technique. The organisms were removed from the agar with saline using a glass spreader, then centrifuged in the cold and washed three times with 200 ml. volumes of redistilled water. The organisms were finally suspended in redistilled water to a concentration of from 50 to 70 mg. dry weight per ml. and stored in the deep freeze. Yields ranging from 0.6 g. to 1.0 g. dry weight per tray were obtained depending on the size of the inoculum. Although longer growth periods resulted in larger yields, these organisms were much less active in incorporation experiments.

B. Labeled Amino Acids

L-leucine, L-histidine, and L-lysine, all labeled with C^{14} in the carboxyl group, were prepared by Dr. P. H. Lowy (49, 75). Glycine-2-C^{14} was purchased from Tracerlab, and L-tyrosine, L-arginine, and L-glutamic acid, uniformly labeled with C^{14}, were purchased from Nuclear Instrument and Chemical Corporation. Unless otherwise noted, the specific radioactivity in counts per minute per millimicromole of the amino acids used was as follows: L-leucine, 4.97; glycine, 6.17; L-lysine, 1.55; L-histidine, 4.50; L-arginine, 5.68; L-tyrosine, 7.72; L-glutamic acid, 5.62.

C. Incorporation Assay

The following experimental procedure for incorporation assays was used unless otherwise noted. Incubation mixtures of 3 ml. were shaken aerobically in 20 ml. beakers in a Dubnoff apparatus (76) at 38° C. All additions were made in the cold, the bacterial preparation being added last. The reaction was stopped by addition of 7.0 ml. of 10% trichloroacetic acid (TCA). The suspension was then transferred to centrifuge tubes. The precipitate was washed 3 times with 7% TCA, heated for 15 minutes at 90° C. with 5% TCA to remove nucleic acid (77), washed 3 times with 7% TCA, washed twice with acetone, and washed twice with ether. Volumes of about 10 ml. were used for each washing. The protein was freed of ether by warming at 50° C. with agitation. A fine powder usually resulted after this operation. The protein samples were then transferred to weighed, circular aluminum planchets (diameter: 19 mm.; depth: 3 mm.). The samples counted ranged in weight from 10 to 20 mg. The sample was uniformly distributed over the planchet surface by agitating with 10 drops of benzene. The benzene was allowed to evaporate at room temperature and the samples were then dried at 80° C., weighed and counted. The radioactivity was determined with an end-window type Geiger counter. The time of counting was usually 30 minutes which, for the radioactivities determined, resulted in a percentage probable error of less than 5%. All counts were corrected to zero thickness.

D. Enzymes

Lysozyme and ribonuclease (RNAase), both crystalline products, were purchased from Armour Laboratories; crystalline desoxyribonuclease (DNAase) was purchased from Worthington Biochemical Sales Co. RNAase and DNAase were stated to be preparations free of proteolytic activity.

E. Solutions

All solutions were prepared with redistilled water. The composition of two solutions generally used in the preparation of extracts were as follows.

1. SDS: The final concentration of the components was 0.712M sucrose, .0615M succinate (Na) pH 6.5, .033M NaCl. This solution will hereafter be referred to as "SDS".

2. KCl-TRIS: 0.8M in KCl and .01M in tris-(hydroxymethyl)amino-methane, pH 7.5 (Cl^-). This solution will be referred to as "KCl-TRIS".

F. Nitrogen Determinations

Protein nitrogen determinations were performed by a direct Nesslerization technique. For determination of the precipitable nitrogen of the KCl-supernatants (See Figure 7) an 0.1 ml. aliquot was precipitated with 3.0 ml. of 7% TCA. The precipitate was kept overnight at 5° C. and then centrifuged and allowed to drain. The precipitate was refluxed with 0.2 ml. concentrated H_2SO_4 for 1 1/2 hours, and clarified with H_2O_2. The solution was then diluted to 30 ml. with H_2O and 5 ml. Nessler's reagent added. The color was read on a Coleman spectrophotometer at 490 mμ .

IV. RESULTS AND DISCUSSION

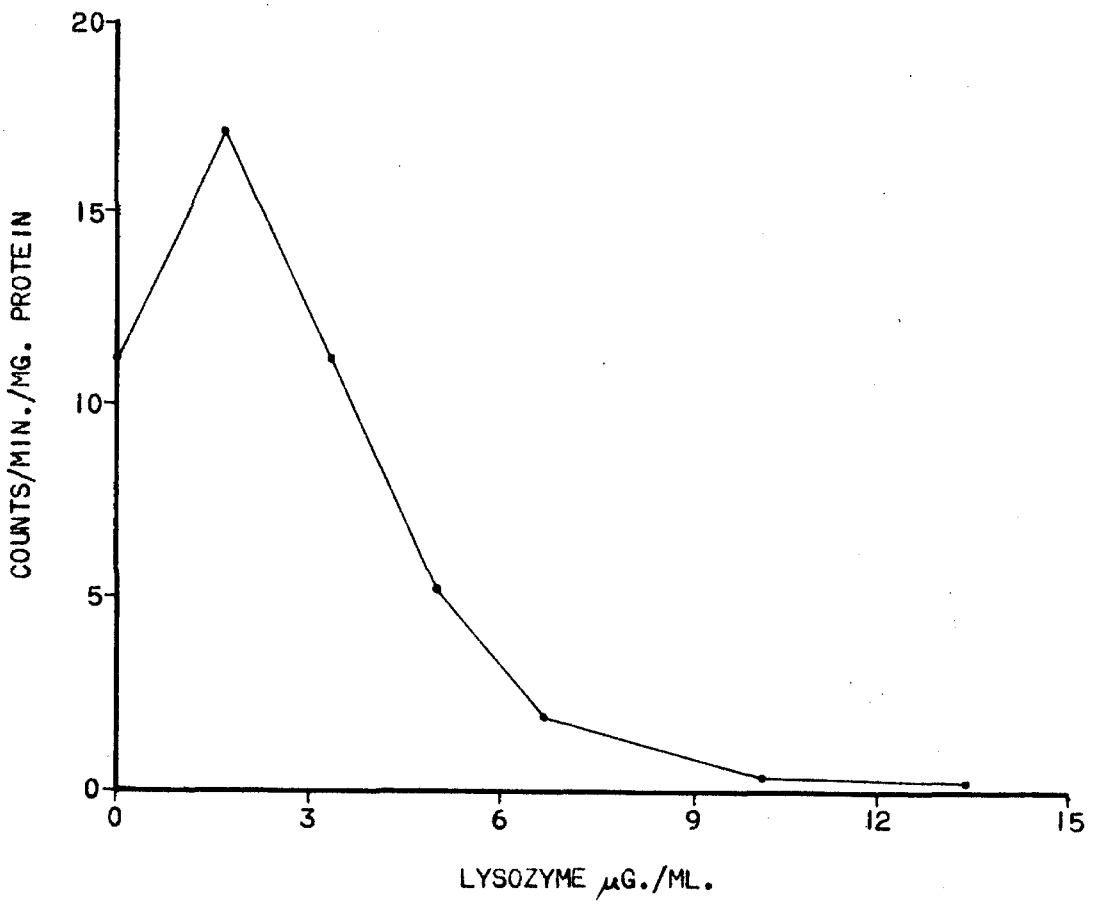
A. Effect of Lysozyme on Respiration and Incorporating Activity

Cells of Micrococcus lysodeikticus were found to incorporate leucine into the protein. The effects of lysozyme on this incorporation and on the endogenous oxygen uptake were studied. It can be seen from Figures 1 and 2 that at lysozyme concentrations much above 10 μg./ml. the incorporating activity and the endogenous respiration were practically abolished. A slight stimulation of both incorporation and respiration was always observed in the same low range of lysozyme concentrations. It was thought in view of the lowered endogenous respiration that the lack of incorporating activity in the lysed cells might be due to dilution of co-factors or to impairment of energy-donating reactions. With this in mind, an attempt was made to reactivate these lysates by addition of many metabolites, co-enzymes, vitamins, kochsafts, etc. in various combinations. In no case was the slightest amount of reactivation observed.

B. Effect of Sucrose on the Incorporating Activity of Lysed Cells

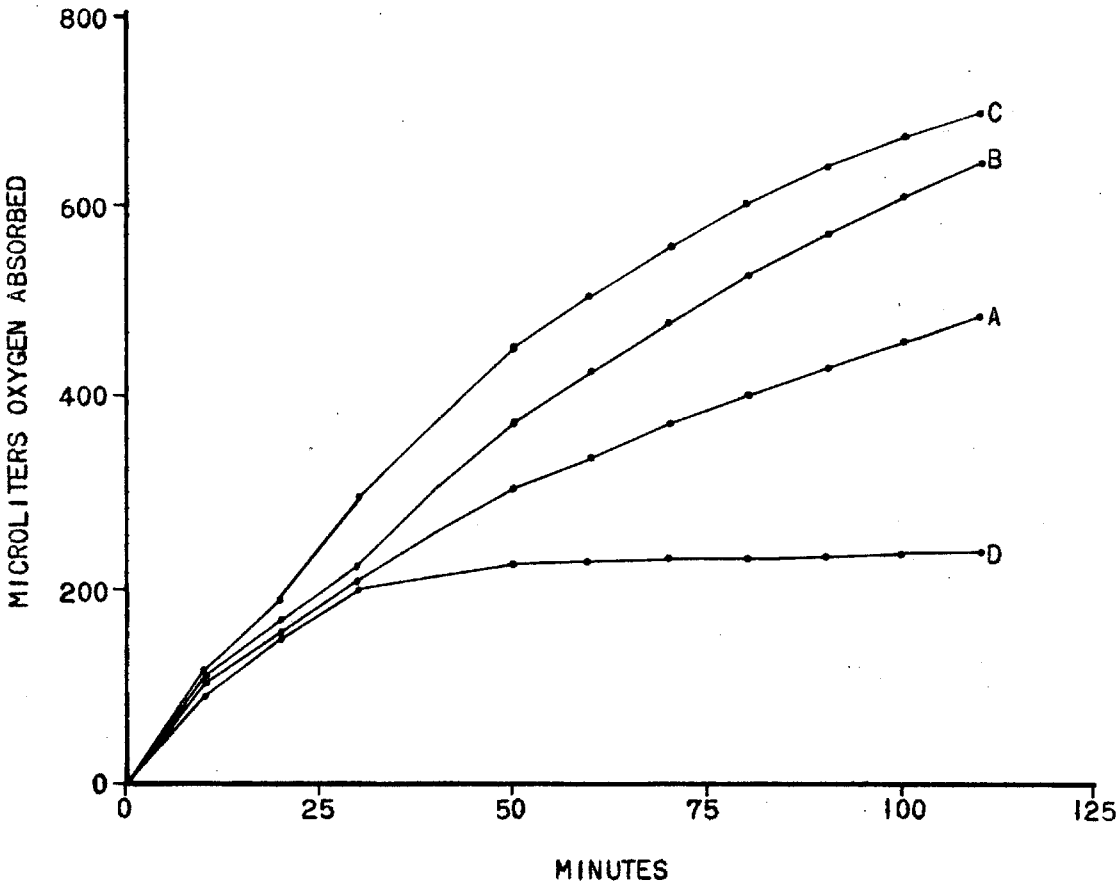
Another explanation for the destruction of activity by lysozyme was that when the cell wall was depolymerized, the systems responsible for the incorporation became very dependent on osmotic conditions. Therefore, the effect of sucrose on the lysate activity was investigated. It was observed that the addition of sucrose during lysis resulted in maintenance of a considerable portion of the activity (Table 2). The effect of sucrose was not significantly changed by using amounts of

Figure 1. Effect of Lysozyme on Whole Cell Incorporation



Each reaction mixture contained 80 mg. of cells, 85.5 μmoles of NaCl, 160 μmoles of succinate (Na) buffer, pH 6.5, 4.33 μmoles of L-leucine-C^{14} (2.58 counts/min./mμmole); lysozyme additions as indicated. Incubated 30 minutes without leucine addition; leucine then added and mixture incubated for 2 hours.

Figure 2. Effect of Lysozyme on the Endogenous Oxygen Uptake of M. lysodeikticus Cells



Each reaction mixture contained 80 mg. cells, 160 μmoles phosphate (Na, K) buffer, pH 6.5, 85.5 μmoles NaCl. Center well contained 0.2 ml. 5N KOH. Final volume 3.2 ml. Temperature 37° C. Usual Warburg technique employed. Lysozyme additions (at zero time) were as follows (μg./ml.): A, none; B, 1.67; C, 3.33; D, 13.3.

Table 2. Effect of Sucrose and Lysozyme on the Incorporation Activity of Whole Cells

Treatment	Activity (Counts/Min./Mg. Protein)
None	29.6
200 μg. lysozyme	0.07
0.48M sucrose	7.5
0.48M sucrose + 200 μg. lysozyme	1.5
0.48M sucrose + 400 μg. lysozyme	0.9
0.48M sucrose + 800 μg. lysozyme	1.0
0.64M sucrose + 200 μg. lysozyme	6.1
0.64M sucrose + 400 μg. lysozyme	5.3
0.64M sucrose + 800 μg. lysozyme	5.6

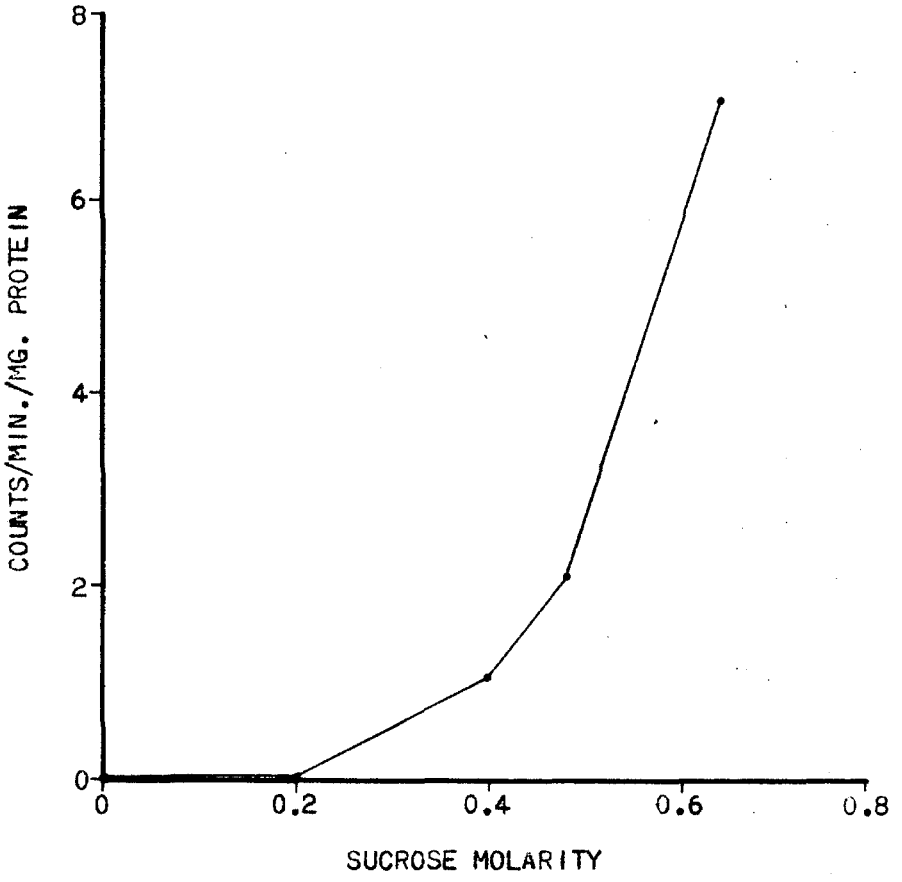
The reaction mixture contained 78 mg. cells, 85.5 μmoles NaCl, 160 μmoles succinate (Na) buffer (pH 6.5), 4.83 μmoles leucine-C^{14}. Sucrose and lysozyme additions as indicated. Incubated 30 minutes in the absence of leucine; incubated 2 hours after leucine addition.

lysozyme twenty times greater than that required for the elimination of activity in the absence of sucrose. It may also be seen that sucrose greatly inhibited the activity of intact cells. No significant effect was ever observed below a concentration of .25M sucrose. Raising the sucrose concentration from .4M to .6M resulted in a six-fold increase in activity (Figure 3). No concentrations above .65M sucrose were investigated because of the difficulty of preparing and pipetting concentrated sucrose solutions. The stock sucrose solutions used were 2.4M, and addition of small volumes was achieved by the use of a semi-automatic syringe. A study of the course of incorporation with time indicated that the sucrose effect manifested itself throughout the course of a two-hour incubation (Figure 4). There was no evidence of a falling off in rate as might have been expected if the sucrose effect were due to only a slowing down of lysozyme activity. If anything, a slight increase in rate has been observed.

C. Effect of RNAase and DNAase on the Sucrose-lysate Activity

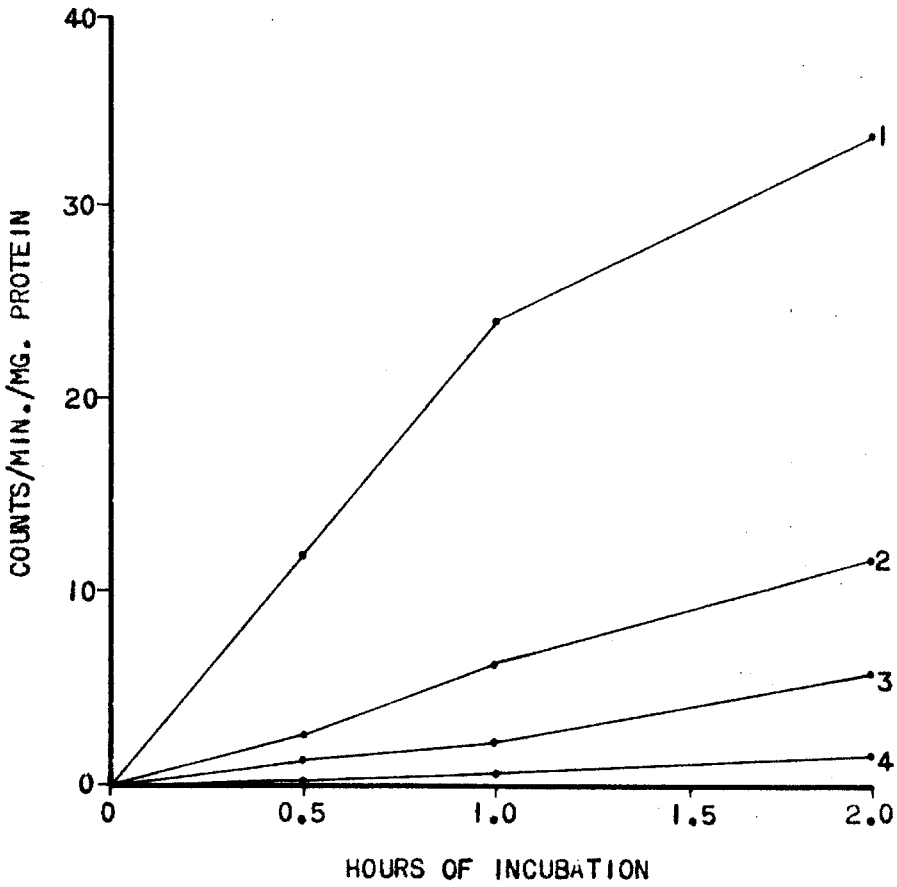
Although incubating the sucrose-lysate on nutrient agar gave no evidence of viable cells, some qualitative difference was sought to indicate that the activity observed was in no way due to the presence of a small number of unlysed cells. The effects of RNAase and DNAase provided evidence in this direction. From the results presented in Table 3 it can be seen that RNAase and DNAase had no effect on the activity of intact cells nor did these enzymes reactivate cells lysed in the absence of sucrose. There was, however, a striking effect of these enzymes on the sucrose-lysate activity. Treatment with DNAase

Figure 3. Effect of Sucrose on the Incorporation Activity of Lysed Cells



Each reaction mixture contained 79 mg. of cells, 85.5 μmoles of NaCl, 160 μmoles of succinate (Na) buffer (pH 6.5), 4.83 μmoles of L-leucine-C^{14}, 0.4 mg. lysozyme. Incubated 30 minutes without leucine; leucine added and mixture incubated 2 hours. Sucrose additions as indicated.

Figure 4. Time Course of the Incorporation



Each reaction mixture contained 76 mg. cells, 85.5 μmoles NaCl, 160 μmoles succinate (Na) buffer (pH 6.5), 0.2 mg. lysozyme (where added), 4.83 μmoles L-leucine-C^{14}. Pre-incubation 30 minutes without leucine. Leucine added and mixtures incubated for indicated times. Curve 1: intact cells. Curve 2: intact cells, .48M sucrose. Curve 3: lysozyme, .64M sucrose. Curve 4: lysozyme, .48M sucrose.

Table 3. Effect of DNAase and RNAase on the Activity of Intact Cells, Lysate, and Sucrose-lysate

Treatment	Activity (Counts/Min./Mg. Protein)
None (Intact cells)	23.2
$MgSO_4$, DNAase	23.6
RNAase	24.5
Lysozyme, $MgSO_4$, DNAase	0.05
Lysozyme, RNAase	0
Sucrose, lysozyme	1.6
Sucrose, lysozyme, $MgSO_4$	2.0
Sucrose, lysozyme, $MgSO_4$, DNAase	6.1
Sucrose, lysozyme, RNAase	0.4

Each reaction mixture contained 78 mg. cells, 160 μmoles succinate (Na) buffer, pH 6.5, 85.5 μmoles NaCl, 4.83 μmoles L-leucine-C^{14}. Other additions where indicated were .565M sucrose, 17.5 μmoles $MgSO_4$, 15 μg. DNAase, 0.7 mg. RNAase, 0.2 mg. lysozyme. Final volume was 3.4 ml. Preincubated for 30 minutes in absence of leucine. Leucine added and incubation carried out for 2 hours. In all beakers the pH was 6.5 ± .1 at the end of the incubation.

increased the activity; RNAase treatment greatly diminished the activity. It was very occasionally (and unexplainably) observed that DNAase had no effect. It might be admitted that this evidence alone does not completely rule out the participation of intact cells; it could be argued that the effect of the enzymes was due to material produced from products of the lysis of only a part of the cells. Other evidence for a distinction between these systems will be presented in a later section.

The production of very viscous material from lysis of dense suspensions of M. lysodeikticus has been previously observed (78). The sucrose-lysate was so viscous as to almost preclude its pipetting. Treatment of the sucrose-lysate with DNAase produced a large drop in viscosity, indicating that the viscous material is largely desoxyribonucleic acid. A similar conclusion with identical evidence was reached independently by Frisch-Niggemeyer (79). DNAase was used routinely in the preparation of sucrose-lysates used in subsequent fractionation procedures.

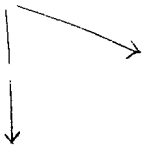
D. Fractionation of Activity in the Sucrose-lysate

Since the sucrose-lysate contained a considerable amount of sedimentable material, it was of interest to see how the activity was distributed between the sedimentable and soluble fractions. A typical fractionation scheme is given in Figure 5. High speed centrifugation of the sucrose-lysate yielded a clear yellow supernatant and a brownish, densely packed pellet. The sucrose-lysate which was prepared in the presence of DNAase in addition had a loosely packed layer of material above the hard-packed pellet. This layer was not unlike the fluffy

Figure 5. Flow Sheet of a Typical Fractionation of Sucrose-lysate*

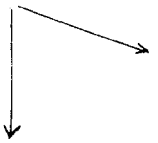
Initial incubation mixture: 0.71 g. cells, 770 μmoles NaCl, 1.8 mg. lysozyme, 0.75M sucrose, 0.055M succinate (Na) buffer, pH 6.5. Final volume 26.1 ml. Incubated 90 minutes at 37° C. This stage is called sucrose-lysate (For a DNAase-treated sucrose-lysate 225 μg. DNAase and 158 μmoles $MgSO_4$ were added to the components mentioned above.)

Centrifugation: Centrifuged at 20,000 R.P.M. (Spinco No. 30 rotor) for 25 minutes.



Supernatant ["Super-1" or Super-1 (DNAase)]

Sediment: ("Original Sediment"). Suspended with 25 ml. SDS solution and centrifuged as above.



Supernatant ["Super-2" or Super-2 (DNAase)]

Sediment: ("Sediment W1X"). Washed as above yielding "Sediment W2X", "Sediment W3X", etc. For assay purposes the final sediment was suspended with 3.0 ml. SDS solution and 1.0 ml. aliquots of this suspension were used for each assay beaker.

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*All operations were performed in the cold unless otherwise stated.

layer described in the preparation of liver mitochondria (80). Several conclusions emerge from the data presented in Table 4, compiled from four representative experiments. Only sedimentable fractions had any activity. In no experiment did a supernatant fraction have any activity when incubated alone. Whereas intact cells lost no activity after repeated washing, the sedimentable fractions of the sucrose-lysate did lose activity. The activity of the sedimentable fractions washed with SDS solution was considerably below that of the original sucrose-lysate. It may also be seen that the lowering of activity due to washing was considerably more effective when the sucrose-lysate was prepared in the presence of DNAase. An attempt was made to restore this activity by adding back supernatants to the sedimentable fractions. Supernatant fractions about doubled the activity of the sediments prepared from DNAase-treated sucrose-lysates. These supernatants caused only a slight decrease in the activity of sediments prepared from non-DNAase treated sucrose-lysates. Although the absolute activities of the DNAase sediments were rather low, the stimulatory effect of the supernatant fractions was well above the probable counting error.

E. Effect of KCl on the Incorporating Activity of Lysates and Intact Cells

An attempt was made to see whether sucrose could be replaced by a suitable amount of an electrolyte which would be more convenient experimentally. The effect of KCl was studied with regard to its ability to maintain sediment activity. The preparation of sediments in the presence of various amounts of KCl is given in Figure 6. The result of an

Table 4. Fractionation and Effects of Prior Treatments on the Activity of Various Preparations

Fraction and Treatment	Activity (Counts/Minute/Mg. Protein) Expt. 23B	Expt. 28A	Expt. 31A	Expt. 32A
Intact cells	21.7	27.4	24.0	
Intact cells washed twice	22.3			
Sucrose-lysate		8.2	10.6	4.6
Original sediment		3.9		
Sediment W1X		3.0	3.4	1.9
Sediment W2X		1.4		
Sucrose-lysate (DNAase)			6.5	8.2
Sediment W1X (DNAase)			0.5	0.4
Sediment W1X (DNAase) + 0.4 ml. super-1 (DNAase)			0.7	
Sediment W1X (DNAase) + 0.8 ml. super-1 (DNAase)			0.9	
Sediment W1X + 0.4 ml. super-1 (DNAase)			2.4	
Sediment W1X + 0.8 ml. super-1 (DNAase)			2.7	
Sediment W1X + 0.4 ml. super-2				1.6
Sediment W1X + 0.8 ml. super-2				1.7
Sediment W1X (DNAase) + 0.4 ml. super-2 (DNAase)				0.8
Sediment W1X (DNAase) + 0.8 ml. super-2 (DNAase)				0.8
Super-1 alone				0
Super-2 alone				0

Preparations as described in Figure 5. Incubated 2 hours with leucine-C^{14}. All assay mixtures containing sediments were 0.64M in sucrose.

Figure 6. Flow Sheet for Preparation of KCl-Sediments*

Sucrose-lysate: The following mixture was incubated for 1 hour at 35° C. with shaking in a 500 ml. erlenmeyer flask. 1.8 g. cells (dry weight), 4.8 mg. lysozyme, 0.6 mg. DNAase, 5.65 X 10^{-3}M $MgSO_4$, 0.7M sucrose, .028M NaCl, .052M succinate (Na) buffer, pH 6.5. Final volume 74.4 ml.

↓

Centrifugation: 5.5 ml. aliquots were distributed among 10 Spinco tubes. Centrifuged at 30,000 R.P.M. (Spinco No. 40 rotor) for 30 minutes.

↓ Supernatants Discarded

Sediments: Suspended with 4.0 ml. of solutions containing various strengths of KCl (from 0.1M to 1.0M); (all solutions were .01M in TRIS buffer, pH 6.5)

Centrifuged at 30,000 R.P.M. for 30 minutes.

↓ Supernatants Reserved for protein nitrogen determinations and assay

Sediments: Each sediment suspended with 0.9 ml. of the appropriate KCl-TRIS solution. 1.0 ml. aliquots taken for each assay beaker. Yields "Sediment W1X .1M KCl", "Sediment W1X .2M KCl", etc.

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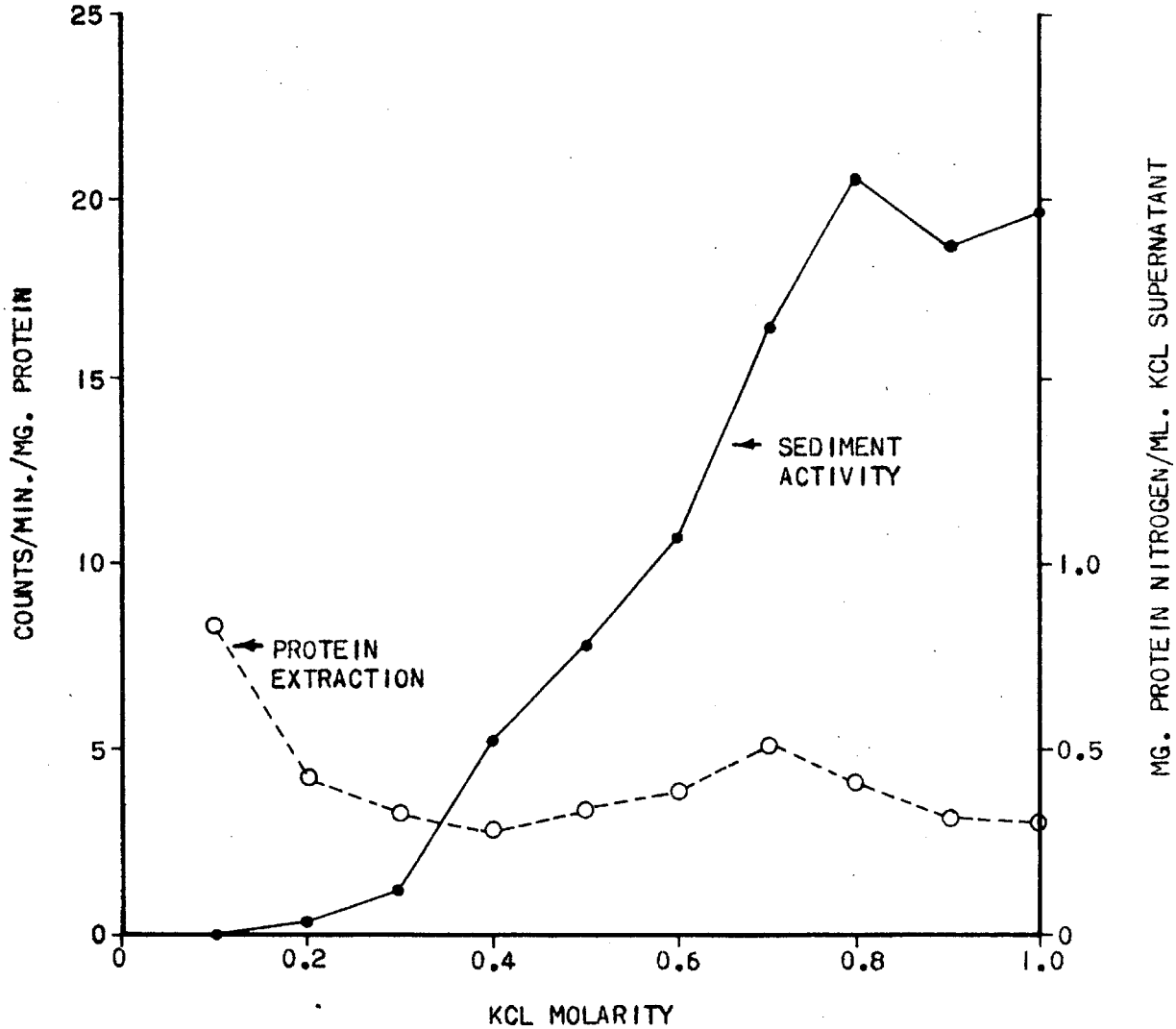
*All operations performed in cold unless otherwise stated.

incorporation assay on these sediments is given in Figure 7. It can be seen that KCl effectively replaced sucrose in maintaining activity. Indeed the activities observed are much higher than in the sucrose experiments. About 0.8M KCl gave maximum activity. When a sediment from a sucrose-lysate was treated with water, practically all the material was rendered soluble. An attempt was made to see what quantitative relation existed between the effectiveness of KCl in maintaining activity and in preventing solution of the sediments. Toward this end the TCA precipitable nitrogen was measured in the supernatants resulting from the first KCl washing (See Figure 6). The KCl did indeed lower the soluble nitrogen content of these supernatants, but the greatest increase in activity was observed at high KCl concentrations where the nitrogen extraction was relatively unchanged. It was concluded, therefore, that this effect of KCl was not solely due to maintaining the integrity of the particulate matter. An essentially similar result was obtained by preparing a sediment in .8M KCl and assaying this at lower KCl concentrations (Figure 8).

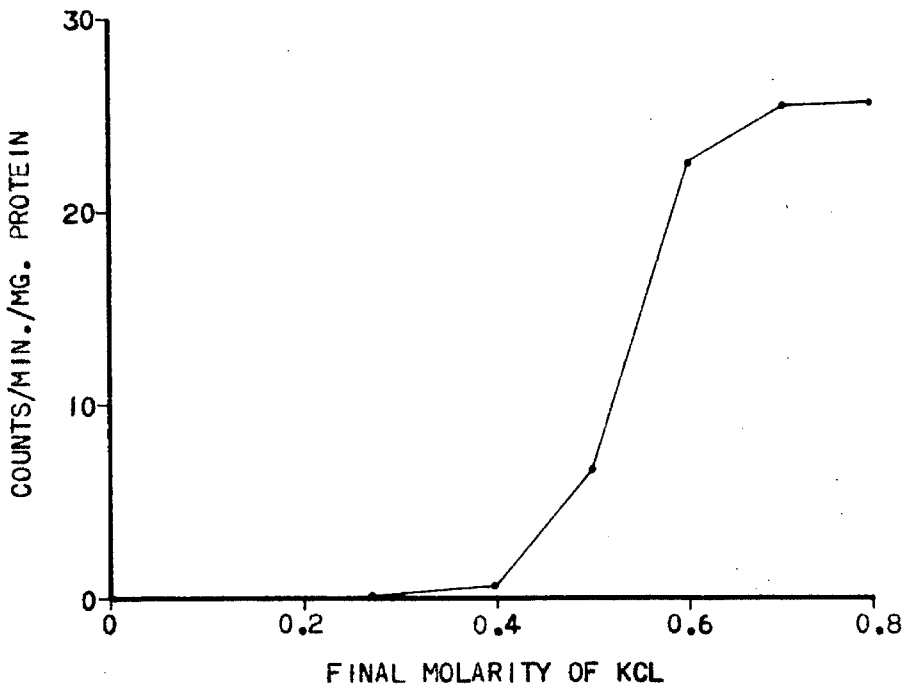
The effect of adding back the KCl supernatants to the sediments was studied at various KCl concentrations. The results described in Figure 9 showed that these supernatants had an inhibitory effect, the relative amount of inhibition decreasing with increasing KCl concentration. There are at least several explanations of the cause of low activity of the sediments prepared with low KCl concentrations. Active material or necessary co-factors may have become soluble, or higher KCl concentrations were necessary in the assay medium, or possibly some irreversible damage was done to the sediment material. An experiment

Figure 7. Effect of Washing and Incubation with Various Concentrations of KCl on the Activity of the Sucrose-lysate Prepared in the Presence of DNAase



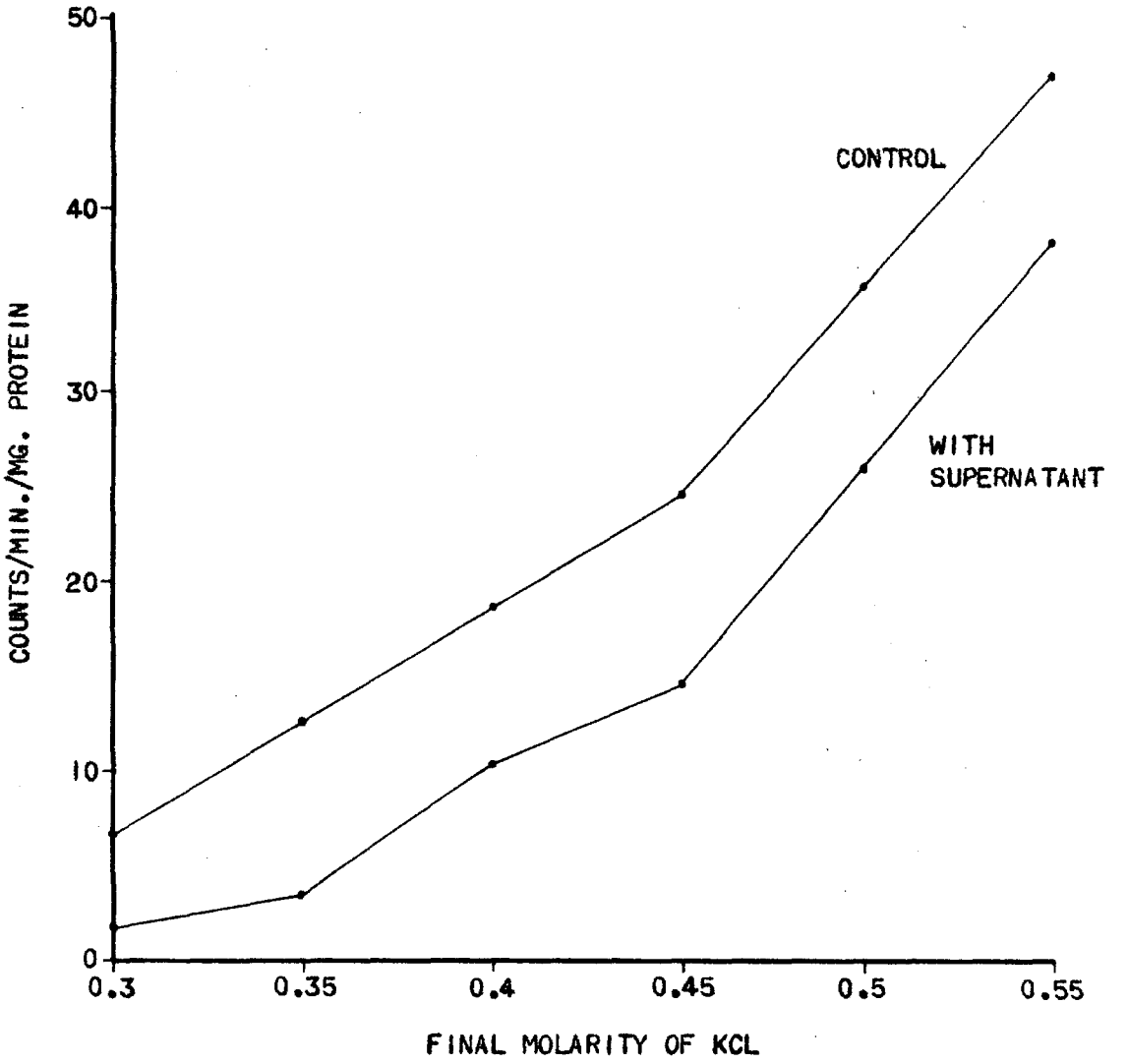
For the incorporation assay each mixture contained 1.0 ml. of the sediment suspension, 200 μmoles TRIS buffer, pH 6.5, 4.83 μmoles leucine-C^{14}. The abscissa refers to the KCl concentration of the washing solution and the incubation assay medium. Final volume 3.0 ml. Incubated 2 hours at 38° C. Preparation of sediments as in Figure 6. TCA precipitable nitrogen determinations as in materials and methods section.

Figure 8. The Activity of a Sediment Prepared in .8M KCl and Incubated at Lower KCl Concentrations



Each reaction mixture contained 1.0 ml. sediment-WLX .8M KCl (prepared as in Figure 6), 300 μmoles TRIS, pH 7.5, 6 μmoles leucine-C^{14}. Final KCl concentration as indicated. Final volume, 3.0 ml. Temperature 38° C. Incubated 2 hours.

Figure 9. Effect of the Supernatant Fraction on the KCl-Sediments Prepared and Incubated at Various KCl Concentrations



Each reaction mixture contained 1.0 ml. of the sediment suspension, 200 μmoles TRIS buffer, pH 6.5, 4.83 μmoles leucine-C^{14}. 1.0 ml. of the appropriate supernatant solution added where indicated. Final KCl concentration as indicated. Preparation of sediment and supernatant material as in Figure 6. Final volume 3.0 ml. Temperature 38° C. Incubated 2 hours.

was performed to test some of these possibilities and the results are given in Table 5. The results showed that the sediment and supernatant fractions prepared with 0.1M KCl were completely inactive when assayed in either 0.1M KCl or .8M KCl; furthermore, combination of the two fractions resulted in no activity at these two KCl concentrations. It was concluded that the loss of activity is irreversible, and that the damage done in 0.1M KCl was probably due to structural disorganization.

The effect of KCl on the incorporating activity of intact cells was also studied. The situation was strikingly different from that observed with the lysates. Here KCl strongly inhibited incorporation by intact cells at concentrations that promoted incorporation in the lysate system (Figure 10). This is further evidence against the participation of unlysed cells in the incorporation observed in the lysate systems.

F. Effect of KCl on the Endogenous Oxygen Uptake of Lysates and Intact Cells

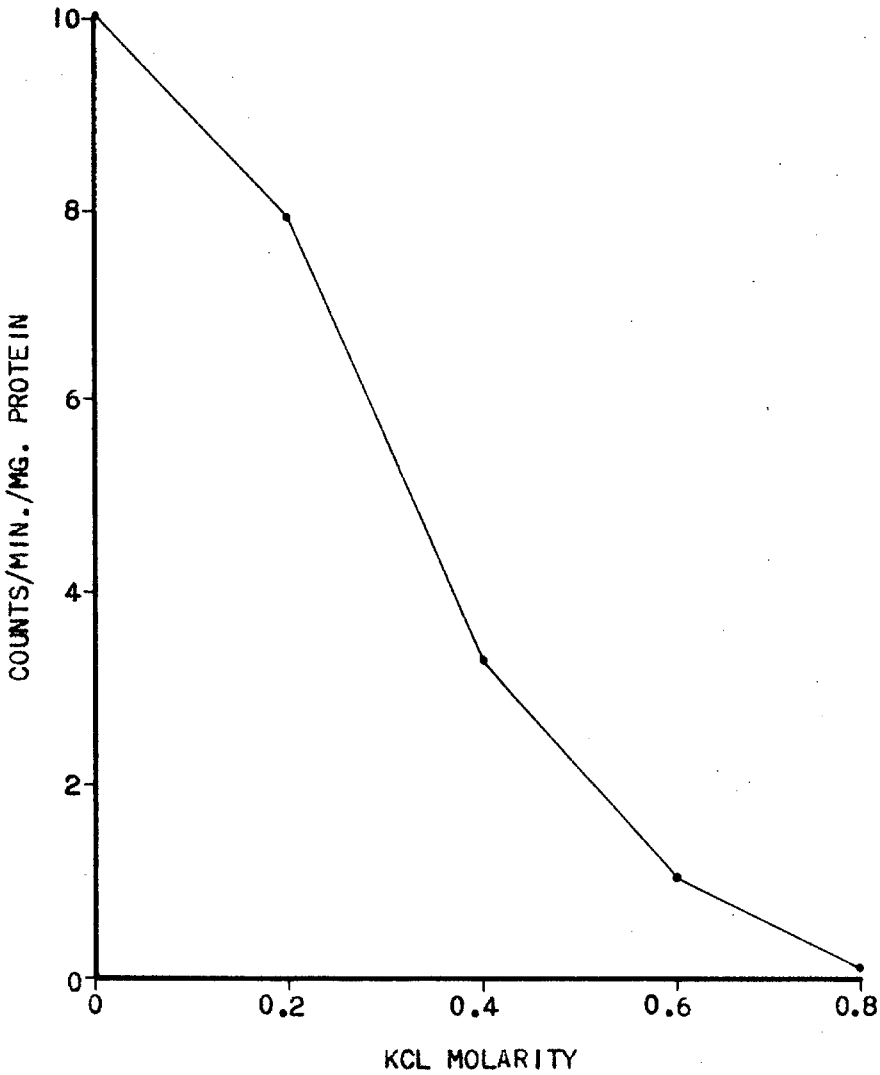
The endogenous oxygen uptake of intact cells and KCl-sediments was measured as a function of KCl concentration. These results are presented in Figure 11 and are compared with the KCl-incorporation effects already mentioned. The respiration and incorporation activities of the sediments responded in a similar manner to changes in KCl concentration. The respiration of the cells was relatively insensitive to high KCl concentrations and it would seem, therefore, that the inhibition of incorporation by KCl was unrelated to the endogenous oxygen uptake *per se* of the intact cells. There could be a number of possible explanations for the parallel observed between respiration and incorporation

Table 5. Irreversible Inactivation of Activity of Sediments Prepared in 0.1M KCl

Fractions Incubated	Final KCl Molarity in Incubation Medium	Activity (Counts/Min. Mg. Protein)
Sediment W1X .8M KCl	.8	18.7
Sediment W1X .1M KCl	.1	0
Sediment W1X .1M KCl	.8	0
.1M KCl - Supernatant	.1	0
.1M KCl - Supernatant	.8	0
Sediment W1X .1M KCl + .1M KCl Supernatant	.1	0
Sediment W1X .1M KCl + .1M KCl Supernatant	.8	0

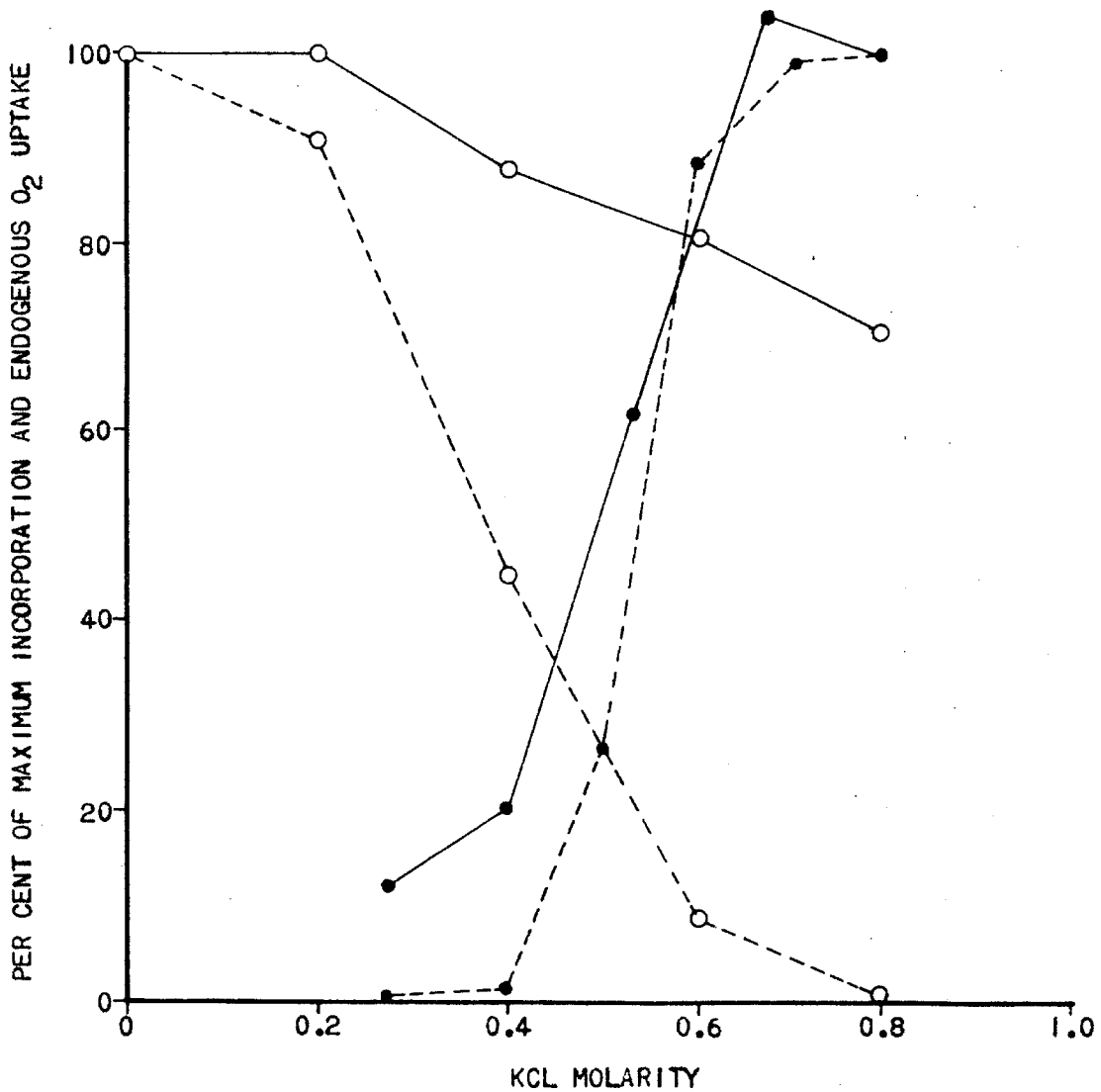
Fractions prepared as in Figure 6. Each incubation mixture contained 1.0 ml. of sediment suspension, 200 μmoles TRIS buffer, pH 6.5, 4.83 μmoles leucine-C^{14}, 1.0 ml. of supernatant where indicated and final KCl concentrations as indicated. Final volume 4.0 ml. Incubated 2 hours at 38° C.

Figure 10. The Effect of KCl Concentration on the Incorporating Activity of Intact Cells



Each reaction mixture contained 76.4 mg. cells, 200 μmoles TRIS pH 7.5, 4.83 μmole leucine-C^{14} (2.6 counts/min./mμmole). KCl additions as indicated. Final volume 3.0 ml. Incubated 2 hours at 38° C.

Figure 11. Effect of KCl on the Endogenous O_2 Uptake and Incorporation Activity of Intact Cells and Sediment WlX KCl



Conditions: (●) Sediment WlX KCl, (○) intact cells, (---) incorporation, (——) respiration.

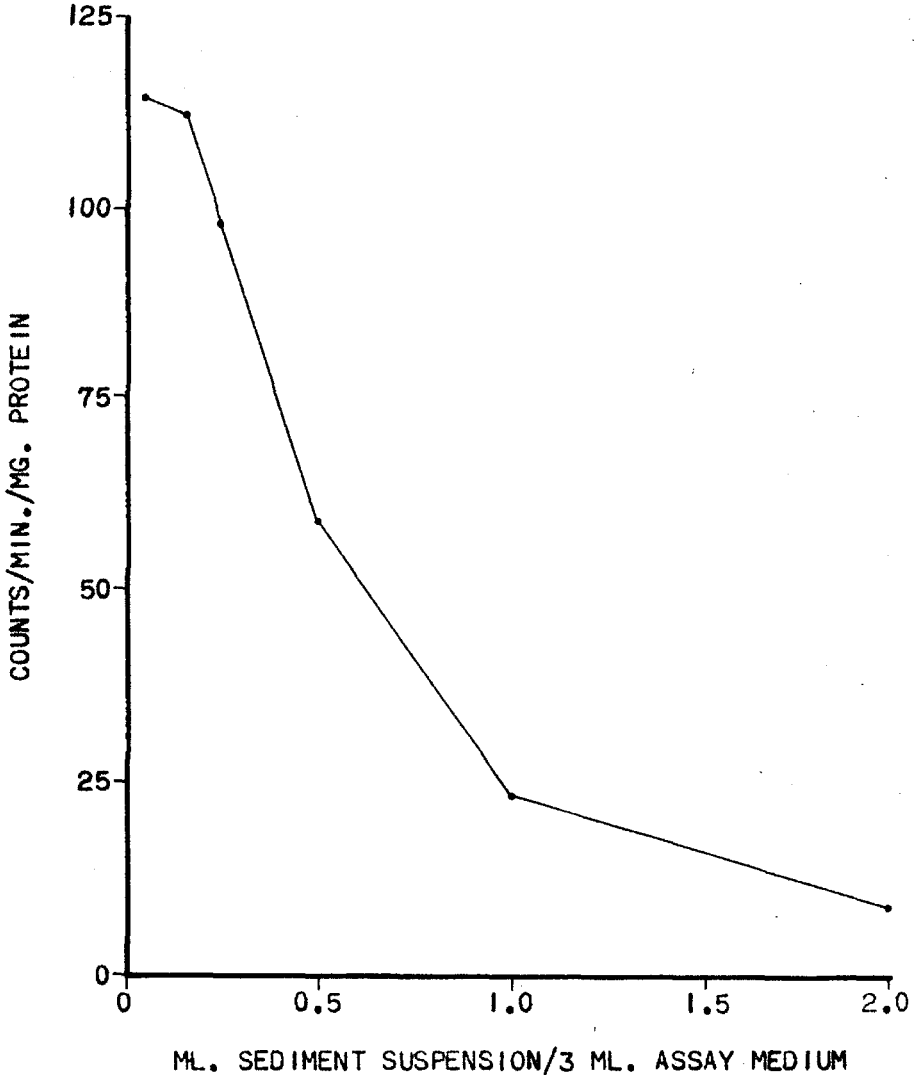
The curves showing the effect of KCl on incorporation have been replotted from the data given in Figures 8 and 10. To measure the oxygen uptake of the sediment preparation, the incubation mixture was the same as in Figure 8. For measuring the oxygen uptake of the intact cells, each incubation mixture contained 80 mg. cells, 300 μmoles TRIS buffer, pH 7.5, and KCl as indicated. Final volume was 3.2 ml. including 0.2 ml. 6N KOH in the center well. The oxygen uptake values were in both cases calculated from the total oxygen taken up in the first two hours. For the cells, 100% = 1 ml. O_2. For the sediment, 100% = .53 ml. O_2.

in the sediment system. It might be that the primary change done was to the respiratory system which caused an inhibition of incorporation by virtue of a lack in energy supply (anaerobic conditions will be shown to lead to inhibition of incorporation). This possibility might be directly checked by attempting to increase the activity of sediments incubated at intermediate KCl concentrations by the addition of more direct energy donors such as ATP. It could also be that the parallel observed was due to some inherent damage which fortuitously affected both systems in a roughly parallel manner.

G. Incorporating Activity as a Function of Sediment Concentration

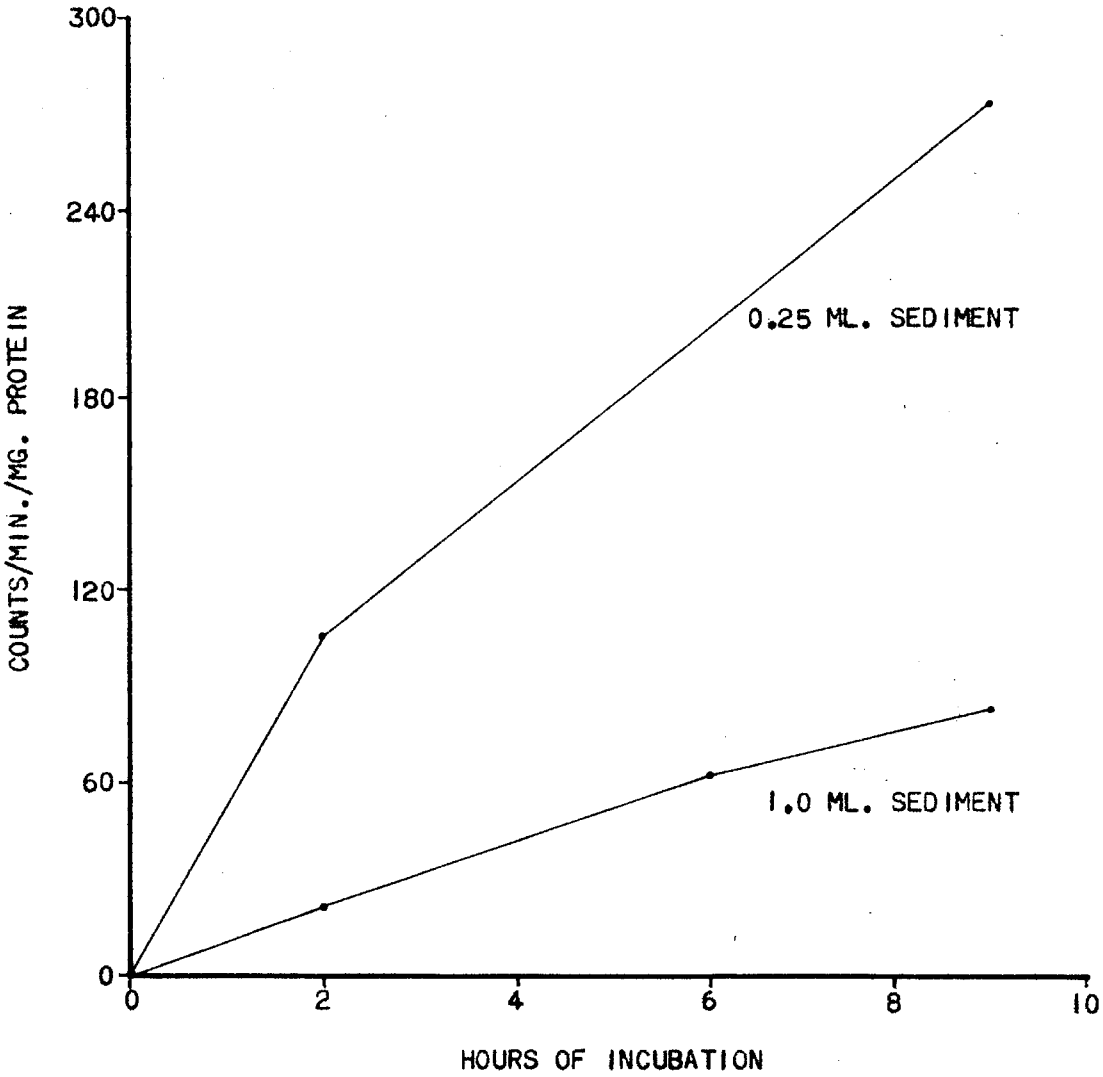
Much effort had been expended in an attempt to fractionate the particulate system by means of differential centrifugation. The results obtained were very erratic, the relative activities of fractions varying from experiment to experiment. At least part of the difficulty can now be attributed to the lack of control over the level at which the various fractions were assayed. The data presented in Figure 12 indicated that the specific incorporating activity and the sediment concentration were inversely related; that is, lowering the sediment concentration in the assay medium resulted in a higher specific activity. This result held up throughout a 9 hour incubation (Figure 13). One explanation of this finding is that at higher sediment levels the radioactive amino acid was limiting. In other words, at low sediment levels there was more radioactive leucine per unit of sediment. This possibility was examined by assaying different levels of sediment at a range of leucine concentrations from 5×10^{-5}M to 5×10^{-3}M. It

Figure 12. Incorporating Activity as a Function of Sediment Concentration in the Assay Medium



Each reaction mixture contained 300 μmoles TRIS pH 7.5, 6 μmoles leucine-C^{14} and was .8M in KCl. The amount of sediment WLX .8M KCl added to each reaction mixture was as indicated. From 1.0 ml. of this suspension 13 mg. of protein was recovered for counting. In order to pipet accurately less than 1.0 ml. amounts of suspension, the suspension was diluted with an equal volume of .8M KCl. (Control experiments indicated that this dilution did not affect the results). To get enough protein to count from mixtures containing less than 1.0 ml. amounts of sediment, at the end of the experiment these were diluted with enough sediment suspension to make a final total of 1.0 ml. of sediment. Incubated 2 hours at 38° C.

Figure 13. The Time Course of Incorporation at Two Levels of Sediment



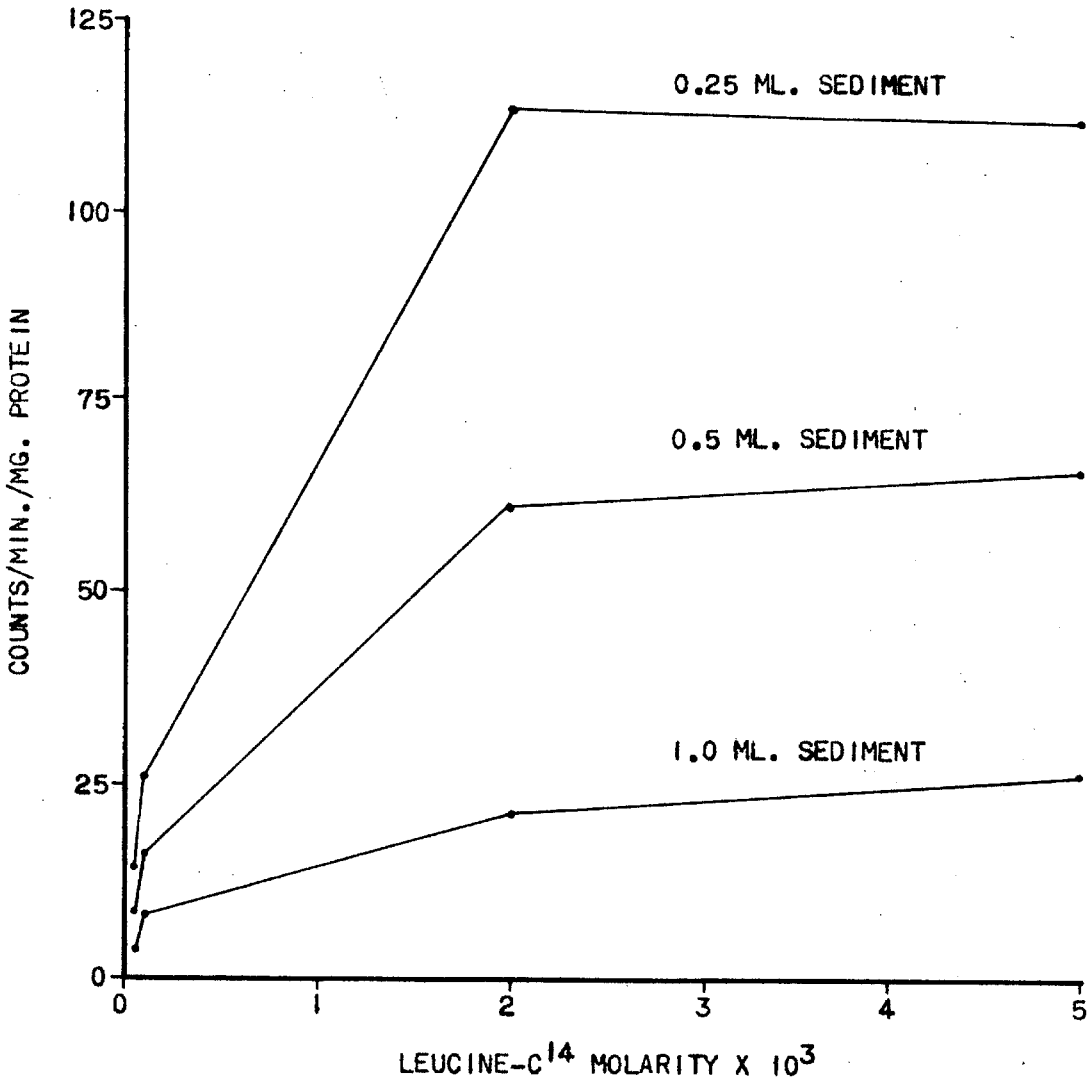
Dilutions handled as in Figure 12. 1.0 ml. of sediment gave 16 mg. protein. Each reaction mixture contained 300 μmoles TRIS buffer, pH 7.5, 6 μmoles leucine-C^{14} and was 0.8M in KCl. Final volume 3.0 ml. Incubated at 38° C. for the times indicated.

was observed (Figure 14) that at leucine concentrations where addition of more leucine gave no significant increase in activity, the sediment concentration phenomenon was observed. Hence, the phenomenon could not be attributed to a limiting leucine concentration. Another possible explanation was that an inhibitor of the reaction was being diluted out, resulting in high activities at high dilutions. Although no direct evidence has been obtained on this point, two observations may be pertinent. An inhibitory effect of supernatants was noted in earlier experiments, albeit a small one at 0.8M KCl (Figure 9). Furthermore, in recent experiments, the concentration effect on occasion has not been quite so marked as indicated in Figure 12. This could possibly be attributed to a slight change in the technique used in preparing sediments. In the earlier experiments, the first sediment was suspended with KCl solution by use of a pipet. In more recent experiments it was convenient to use a syringe in suspending the sediments. This may have done a more thorough job of washing. At any rate this variable must be controlled in any experiments with this system. Better still, the cause of the phenomenon should be understood, so that it may be possibly eliminated. In all experiments reported in this thesis this variable has been intentionally or fortuitously controlled.

H. Incorporation of Amino Acids other than Leucine

All amino acids which have so far been tested have been incorporated into the protein of the particulate system. The relative rates of incorporation (on a molar basis) of the various amino acids are given in Table 6 for a number of experiments. Although a considerable

Figure 14. Specific Activity as a Function of Sediment Concentration and Leucine-C^{14} Concentration



Experimental design as in Figure 12. Leucine additions as indicated. 1.0 ml. of sediment gave 16 mg. protein for counting. Incubated 2 hours at 38° C.

Table 6. Relative Rates of Incorporation of Various Amino Acids

	Relative Incorporation Rate. Glycine = 100						
Experiment No.:	44C	44D	50A	50B	51A	52A	44C
System:	Sediment W1X .8M KC1						Intact Cells
Amino Acid							
Glycine	100	100	100		100		100
Leucine	57	68	54	50*	21	50*	49
Lysine	12	15	18	19	11		50
Arginine			39	37	23		
Tyrosine			16	19	14		
Glutamic Acid			10	11	7		
Histidine			9	9	5	9	
Actual incorporation of glycine (μmoles/gram protein/2 hours)	16.5	3.7	6.2	5.1	22.8	21.8	14.8

Conditions: Each reaction mixture contained 1.0 ml. sediment W1X .8M KC1 (or 61 mg. cells where indicated), 300 μmoles TRIS buffer, pH 7.5. All mixtures except those containing cells were .8M in KC1. All C^{14}-amino acids were 2 X 10^{-3}M. Final volume 3.0 ml. Incubated 2 hours at 38° C.

*In these experiments (where no glycine was used) the leucine value is arbitrarily set at 50.

variation in absolute activities was observed in the experiments cited, it can be seen that the relative rates are roughly constant. These relative rates would appear to be a characteristic of the system studied since a difference in relative rates was observed between intact cells and the lysate system. However, more data on intact cells are needed to establish this point.

I. Incorporating Activity as Affected by Various Compounds and Treatments

Some evidence has already been presented suggesting a relation between respiration and incorporation. Further evidence along this line was the almost complete lack of incorporation in an atmosphere of nitrogen (Table 7). The small amount of residual activity observed under these conditions was very possibly due to the time required for complete elimination of oxygen. Although this is good presumptive evidence of the role of oxidative reactions in driving incorporation, a more cogent result would be the demonstration of an anaerobiosis inhibition reversal by compounds such as ATP. This has not been attempted. Addition of 2,4-dinitrophenol to the incubation mixture was shown to have an inhibitory effect (Table 7). Half maximal inhibition was observed at about 1.7×10^{-3}M, a concentration somewhat higher than that reported for inhibition of incorporation in other systems (44, 48, 49, 58). It has been shown in a variety of systems that dinitrophenols prevent oxidative phosphorylation at concentrations of the order of 10^{-4}M (82). Although no direct proof is available in this system, one might assume that the observed dinitrophenol inhibition was of a similar nature.

Table 7. Effects of Various Compounds and Treatments on the Incorporation Activity of Sediments (WLX .8M KCl)

Treatment	Per Cent of Control Activity
None	100
Anaerobiosis	
Leucine	2
Arginine	6
Lysine	-2
Tyrosine	10
2,4-dinitrophenol 0.5 X 10^{-3}M	91
2,4-dinitrophenol 1.0 X 10^{-3}M	64
2,4-dinitrophenol 1.5 X 10^{-3}M	57
2,4-dinitrophenol 5.0 X 10^{-3}M	10
2,4-dinitrophenol 10^{-2}M	2
Orthophosphate (Na) .017M	74
Sodium polyphosphate 0.1 mg./ml.	92
Sodium polyphosphate 0.5 mg./ml.	32
Sodium polyphosphate 1.0 mg./ml.	15
ATP 10^{-3}M	70
Glucose-1-phosphate (K) 3 X 10^{-3}M	89
5 minutes at 80° C. before assay	.03

All experiments were performed with leucine-C^{14} unless otherwise noted. For the anaerobiosis experiment, the reaction mixtures were in a nitrogen atmosphere throughout the course of the incubation; the controls being incubated in air. The sodium polyphosphate used was prepared according to Chayen et al. (81) and was reputed to be a linear polymer having a molecular weight of the order of 10^5. Other conditions as in Table 6.

All phosphate containing compounds which have been tested (Table 7) have exerted varying degrees of inhibition of incorporation. That compounds such as ATP or glucose-1-phosphate exert no stimulatory effect could very well be in line with the high endogenous respiration, that is, there already exists an optimal endogenous energy supply. On the basis of some experiments with yeast, Chayen et al. postulated that polyphosphate compounds served as an energy supply for protein synthesis (81). A strong inhibitory effect of polyphosphate was observed in the lysate system (Table 7); half maximal inhibition was observed at a polyphosphate concentration equivalent to 10^{-3}M orthophosphate. The inhibitory effects of these phosphate compounds may possibly be due to the binding of some cation essential for activity. As already stated, it would be of interest to determine the effect of these phosphate compounds under anaerobic conditions.

J. The Effect of an Exogenous Amino Acid Supply on Incorporation

Several experiments have been performed to examine the effect on incorporation of adding a mixture of unlabeled amino acids. Although these data are incomplete, several points about these experiments seem worth mentioning. Large stimulations of incorporation have been observed (leucine and histidine) on addition of amino acid mixtures. These stimulations have been greatest when the assays were carried out with low sediment concentrations. Further, the effect of a complete mixture of amino acids cannot be duplicated by separate addition of small groups of amino acids (Table 8). While not enough systematic experiments have been done, it seemed that the greater effects of amino acid

Table 8. Amino Acid Effects on Incorporation of Leucine

Amino Acids Added	Mg. Protein* per 3.0 ml. Incubation Mixture	Activity (% of Control without Amino Acids)
Complete mixture	16	90
Complete mixture	4	440
Arginine + lysine + methionine	4	195
Glutamic acid + aspartic acid + proline + threonine	4	188
Cysteine	4	142
Histidine + glycine + alanine + serine	4	114
Isoleucine + valine	4	107
Phenylalanine + tyrosine + tryptophan	4	83

In addition to the components indicated each incubation mixture contained 300 μmoles TRIS pH 7.5, 6 μmoles leucine-C^{14}, and was .8M in KCl. Final volume 3.0 ml. Incubated 2.5 hours at 38° C. The complete amino acid mixture contained glycine, DL-serine and the L forms of lysine, alanine, valine, glutamic acid, aspartic acid, threonine, tyrosine, tryptophan, histidine, arginine, phenylalanine, methionine, isoleucine, and proline. Where indicated, 3.0 μmoles of DL-serine and 1.5 μmoles of each of the other amino acids were added.

*1.0 ml. of sediment W1X .8M KCl gave 16 mg. protein.

mixtures at low sediment concentrations might be reasonably explained by a dilution of the endogenous amino acid pool, thus making amino acids limiting in the reaction. This result is to be compared to the increase in intrinsic activity observed (without amino acid addition) at low sediment concentrations (Figure 12).

K. The Composition of the Free Amino Acid Pool of Sediments W1X .8M KCl. Implications.

The composition of the free amino acid pool of sediments W1X .8M KCl was determined according to the procedures described by Levy and co-workers (83, 84). Briefly, this method involved converting the amino acids to their dinitrophenyl derivatives, which are yellow in color, and measuring the intensity of the color after these derivatives were resolved by two-dimensional paper chromatography.

Preparation of the protein free extract for analysis was as follows: to 4.0 ml. of a freshly prepared sediment W1X .8M KCl suspension containing 80 mg. of protein was added 1.0 ml. of 35% TCA. After 4 hours in the cold the mixture was centrifuged and the supernatant was reserved. The precipitate was resuspended with 3 ml. of 7% TCA and centrifuged. Both supernatants were combined and were continuously extracted with ether for 15 hours to remove the TCA. This solution was treated with a 6 to 7-fold excess of 1-fluoro-2,4-dinitrobenzene under conditions prescribed by Levy (83); the ether soluble derivatives were extracted from the acidified solution and the subsequent chromatography was as described (84). Control experiments with known amino acid mixtures were performed.

Before presenting the results some comments concerning the method are necessary. The dinitrophenyl amino acids were identified only by comparison with known compounds. Glutamic acid and aspartic acid are poorly separated from each other by this method. The acid soluble derivatives were not analyzed because of the presence of large amounts of KCl which interfered with the chromatographic separations. The recovery of an amino acid from known mixtures ranged from 70 to 100 per cent, except in the case of lysine which was always unexplainably low, ca. 20 per cent. This recovery however is quite sufficient for the purposes of the experiment.

The results of the analysis, calculated as μmoles of amino acid per mg. sediment protein, were as follows:

Glutamic acid + aspartic acid	.055
Alanine	.063
Tyrosine	.037
Methionine	.007
Proline	.005

Many amino acids were not detected at all. These were serine, threonine, glycine, valine, leucine, isoleucine, phenylalanine, and tryptophan. Therefore these amino acids must have been present (if at all) in amounts less than .005 μmoles/mg. protein. No information concerning arginine, histidine and lysine was obtained.

These results are to be compared with the rates of amino acid incorporation observed in this system. For leucine, the rates observed (depending on the sediment concentration) usually ranged from .01 to .02 μmoles leucine incorporated/mg. protein/2 hours. In assays of longer

duration, up to 12 hours, total leucine incorporation has been observed to be as high as .05 μmoles/mg. protein. If it is assumed that the observed amino acid incorporation was due to synthesis of new protein molecules, and that each amino acid represented 5 per cent of these molecules, than about .05 μmoles of each amino acid per mg. sediment protein would be required for this synthesis to occur. The values mentioned in this paragraph are incorporation rates measured in the absence of exogenous amino acids. These rates are from 2 to 4 times higher when the assays are performed in the presence of an exogenous amino acid supply.

Although the data concerning the initial composition of the amino acid pool and the rates of amino acid incorporation lead to no categorical answers concerning the mechanism of amino acid incorporation, nevertheless, these data may perhaps help in defining the problem and in suggesting future experimentation. These data will be discussed in the light of two different (but not mutually exclusive) possible mechanisms of amino acid incorporation in M. lysodeikticus.

One possible view is that incorporation reflects only the net synthesis of new protein molecules from free amino acids. Since only several amino acids were found in amounts comparable to the observed magnitude of incorporation, this explanation seems to be ruled out unless it is further assumed that either proteins of unusual composition were formed or that a concomitant breakdown of endogenous protein occurred, thus yielding a small, but complete and continual, supply of free amino acids. Some evidence in favor of the latter assumption has been obtained by Gorini and Crevier (85) who showed that a

proteolytic system was present in M. lysodeikticus. However, their work was with a completely lysed system and is therefore not directly comparable. It would be advantageous to study the incorporation of amino acids into a single well-defined protein. This particular system offers a unique opportunity for such experiments since crystalline catalase has been isolated from M. lysodeikticus; this enzyme constitutes from 1 to 2 per cent of the dry weight of this organism (78).

Another possible view is that amino acid incorporation reflects a direct exchange reaction (See I, B, 3). This kind of mechanism would not require the presence of a free amino acid pool.

That net synthesis can occur in this system is the most reasonable interpretation of the data which show that amino acid incorporation was stimulated by addition of a mixture of non-labeled amino acids. Under limiting amino acid conditions, however, an exchange mechanism is by no means ruled out.

L. Are Peptide Bonds Formed in the Incorporation Reaction?

Other workers have found that labeling of protein could occur via anomalous reactions which seem not to be concerned with the formation of peptide bonds in a protein (32). Ultimate proof that the reaction measured was actually peptide bond formation would involve isolation and identification of small peptides containing a radioactive amino acid residue. This has not been done in the present work. However, it seems unlikely that the incorporation measured here was anomalous because the system was shown to have some rather normal biochemical

properties such as inhibition under anaerobic conditions and inhibition by heating at 80° C. for 5 minutes (Table 7). Furthermore, the stability of the bond(s) formed was such that they withstood heating at 90° C. with 5% TCA. The radioactive protein obtained from experiments with leucine, lysine and histidine were shown to contain no free labeled amino acid by treatment of the protein with ninhydrin under conditions which would lead to decarboxylation of free amino acids; no significant evolution of radioactive carbon dioxide occurred (Table 9).

An experiment was carried out to determine which amino acid residues of the radioactive protein isolated after incubation with leucine-C^{14} contained the C^{14}. The details of the experiment are given in Figure 15. Briefly, this experiment involved ion-exchange chromatography of the protein hydrolyzate and identification of radioactive fractions. This fractionation gave a single radioactive peak containing all the counts put on the column (Figure 16). Paper chromatography of the material in this peak with three different solvents (listed in Figure 15) showed a single ninhydrin spot which corresponded to the radioactivity in each case. The R_f values of this substance in the three solvents used corresponded precisely with those of known leucine. Carrier recrystallization led to constant specific radioactivity after three crystallizations with overall recovery of 40 per cent of the initial carrier. It was concluded, therefore, that this substance was leucine and that leucine-C^{14} was incorporated into the protein as such.

Although direct proof was not obtained, all the data were consistent with true peptide bond formation.

Table 9. Release of Radioactive Carbon Dioxide from Protein Treated with Ninhydrin

	Labeled Amino Acid Used in Incubation		
	Leucine	Lysine	Histidine
Mg. radioactive protein treated with ninhydrin	18.7	68.1	78.3
Total counts/minute/sample	858	217	656
Total counts/minute released by ninhydrin treatment (measured as $BaCO_3$)	2.1	1.4	4.6

The protein samples used represented radioactive protein pooled from a number of experiments (all with sediments W1X .8M KCl). The ninhydrin treatment was carried out according to the procedures of Van Slyke et al. (86).

Figure 15. Isolation of Leucine-C^{14} from Radioactive Protein

1. HYDROLYSIS

578 mg. protein (26,000 counts/minute) hydrolyzed in a sealed tube with 7.0 ml. 6N HCl at 105° C. for 25.5 hours. Humin filtered by gravity. Solution taken to dryness in vacuo and dissolved with 5.4 ml. 1.5N HCl. This solution contained 18,100 counts/minute.

2. ION EXCHANGE CHROMATOGRAPHY [According to Moore and Stein (87)]

The solution was placed on a 2.2 cm. by 45 cm. Dowex-50 column previously equilibrated with 1.5N HCl. An automatic fraction collector was used to collect fractions containing 225 drops (15 ml.) at a rate of 2 to 3 drops per minute. The eluting solutions were, in the order used: 390 ml. 1.5N HCl, 1250 ml. 2.5N HCl, and 1250 ml. 4N HCl. 0.5 ml. aliquots were dried and counted (See Figure 16).

3. ISOLATION AND IDENTIFICATION

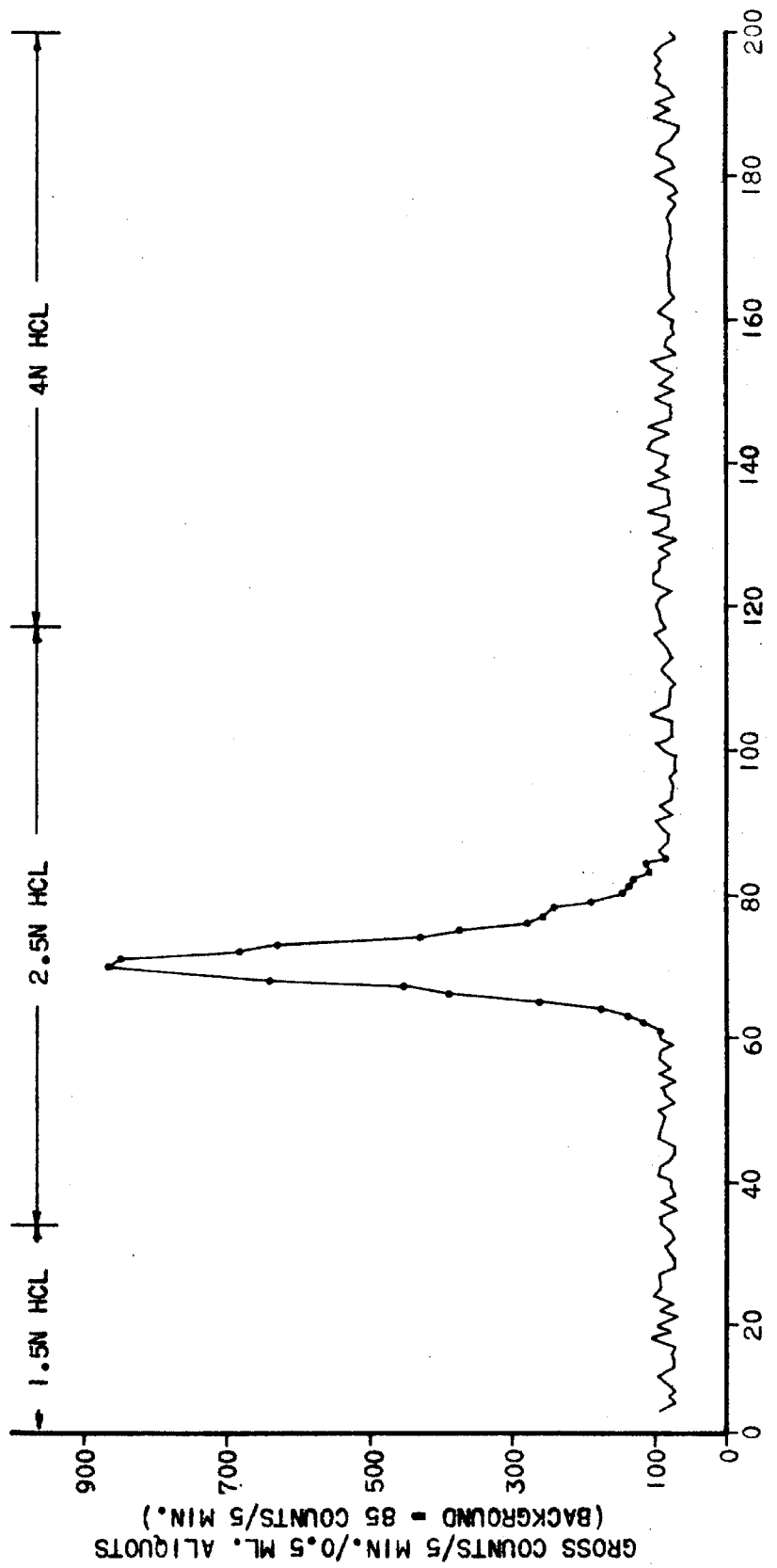
The radioactive fractions were pooled and the solution was taken to dryness in vacuo. Total counts/minute recovered in this peak: 18,900.

a. Paper chromatography in 3 solvents: 5 parts n-propanol: 2 parts 1% NH_4OH, water-saturated phenol, 11 parts n-butanol: 7 parts 4.4N acetic acid.

b. Carrier recrystallization from 70% ethanol.

(See text for results.)

Figure 16. Fractionation of a Radioactive Protein Hydrolyzate on Dowex-50



M. The Problem of Intermediates in Incorporation and the Conversion of Leucine to α-ketoisocaproic Acid

Several experiments were performed in an attempt to determine whether derivatives of leucine-C^{14} other than protein could be identified. Typical experimental details are given in Figure 17. The experiments were designed so that a 1% conversion of leucine-C^{14} to a dialyzable compound could easily be detected, assuming it could be separated from the large excess of leucine-C^{14} already present. It was thought that treatment of the mixture with RNAase might produce material that would not otherwise have been dialyzable. Various reaction mixtures containing leucine-C^{14} were incubated and then dialyzed against water. The dialyzates were concentrated and fractionated with paper electrophoresis. All dialyzates gave two well-defined radioactive zones in identical positions. The major radioactive zone hardly migrated at all from the origin and was presumed to be leucine. The other zone contained a negatively charged compound which migrated from 8 to 15 cm. from the origin depending on the duration of the run. In the experiment mentioned, with intact cells (Figure 17), the ratio of counts in the acidic zone to counts in the leucine region was about the same for the three dialyzates (No. 1, .3; No. 2, .4; No. 3, .3). (In other experiments with sediments W1X .8M KCl under usual 2 hour assay conditions about a 5 to 15% conversion of leucine to the acidic compound was observed). An attempt was made to recover leucine from this compound by acid hydrolysis (6N HCl, sealed tube, 105° C., 20 hours). Only 5 per cent of the counts could be recovered after this treatment. It was then suspected that the

Figure 17. Experimental Procedures Employed in the Isolation and Identification of Ketoisocaproic Acid

1. INCUBATION

Three mixtures were incubated at 38° C., each containing 158 mg. cells, 200 μmoles TRIS buffer, pH 7.5. In addition No. 2 and No. 3 contained 24 μmoles leucine-C^{14} (10 counts/minute/mμmole). Volume, 6.0 ml. After 2 hours incubation 0.4 mg. lysozyme added to each mixture; in addition, 1.4 mg. RNAase added to No. 1 and No. 3, and 24 μmoles leucine-C^{14} added to No. 1. Final volume 7.1 ml. Incubation continued for 2 hours.

2. DIALYSIS

Each reaction mixture dialyzed for 24 hours at 5° C. against 70 ml. water. The dialyzates were lyophilized.

3. ETHER EXTRACTION

No. 2 dialyzate was adjusted to pH 0.8 with HCl and was continuously extracted with ether for 24 hours. The ether was allowed to evaporate at room temperature and the remaining oil was dissolved in 3 ml. water.

4. PAPER ELECTROPHORESIS

Substances applied to 6" X 10" strips of Whatman No. 1 filter paper. Electrophoresis carried out with 0.1M phosphate (Na, K) buffer, pH 6.8. Run at 600 volts for periods of from 2 to 4 hours. The current usually rose from about 5 to 15 milliamperes. The paper was immersed in chlorobenzene throughout the run. The paper was scanned for radioactivity by counting successive 1 cm. sections.

5. CARRIER ISOLATION AS THE 2,4-DINITROPHENYLHYDRAZONE

To 0.3 ml. of the ether extract (3,000 counts/minute) and 0.5 g. sodium α-ketoisocaproate [synthesized according to Metzler et al. (88)] was added 100 ml. of 1% 2,4-dinitrophenylhydrazine (2N HCl). The mixture was heated for 5 minutes at 100° C., cooled, and the product was filtered and washed with cold 2.5N HCl. The product was twice crystallized from water. Yield 0.47 g. m.p. 159-161° C. [lit. 162° C. (89)]. Specific radioactivities: 2.79 and 2.84 counts/minute/mg.

compound might be α-ketoisocaproic acid, loss of counts being due to decarboxylation. This compound was completely extracted from the dialyzates with ether, and the ether soluble material was shown to have the same electrophoretic mobility as synthetic α-ketoisocaproic acid. Carrier isolation of the ether soluble material as the 2,4-dinitrophenylhydrazone derivative resulted in constant specific radioactivity after two crystallizations. It was concluded, therefore, that M. lysodeikticus can convert leucine to α-ketoisocaproic acid. A likely mechanism for this reaction might be transamination with pyruvate or α-ketoglutarate.

This negative experiment of course does not exclude the participation of intermediates, either free or bound, in the incorporation of leucine into protein.

N. Summary and Final Discussion

A brief summary of some of the results obtained will be given in this section. These results will be discussed in the light of pertinent findings of other workers.

Treatment of Micrococcus lysodeikticus cells with lysozyme in the presence of high concentrations of sucrose has yielded a particulate system, as distinct from intact cells, that carries out the incorporation of amino acids into protein. When the assays were carried out in the presence of high concentrations of KCl instead of sucrose, incorporating activity was as fast or faster than in intact cells. The system was sedimentable, had a high endogenous respiration, and required the continued presence of high sucrose or KCl concentrations

for activity. When the KCl concentration was progressively lowered, respiration and incorporation decreased at parallel rates. In contrast to the lysate system, the incorporating activity of intact cells was abolished at high KCl concentrations. Anaerobiosis, 2,4-dinitrophenol, and RNAase treatment decreased activity. Stimulatory effects were observed by the addition of amino acid mixtures and by DNAase treatment. The activity of these particles was shown to be dependent on the concentration at which they were assayed.

The effect of sucrose in maintaining the activity of lysozyme treated cells and the effects of RNAase and DNAase on activity have been confirmed by Beljanski (58).

The work of Weibull with Bacillus megaterium is pertinent to this discussion (90). Weibull found that when this rod-shaped bacillus was treated with lysozyme in the presence of .2M sucrose, the bacterial cell wall was depolymerized, the rest of the cell remaining intact and appearing now as a spherical body, the protoplast. If the lysis was performed in dilute phosphate buffer only two structural elements were apparent, empty spherical "ghosts" and lipoidal granules. Weibull found that these protoplasts would lyse when the sucrose concentration was lowered. The question remains whether in the case of sucrose-lysates of M. lysodeikticus we have dealt with protoplasts or a mixture of smaller particles. Electron microscopy might decide this point directly. Short of microscopy, the gross observations have been the same. There was a hint that the particulate material was not homogeneous since centrifugation of sucrose-lysates yielded sediments which were composed of at least two distinct layers of material. Sub-cellular particles have been obtained from M. lysodeikticus by crushing a water

suspension of cells with powdered glass (91). These particles participated in the oxidation of tricarboxylic acid cycle intermediates. Freezing these particles abolished this activity.

It is of interest to compare several features of the incorporation studied in several cell-free systems, namely, the particulate system of rat liver investigated by Siekevitz (44) and later by Zamecnik and Keller (45), disrupted cells of Staph. aureus studied by Gale and Folkes (62) and the M. lysodeikticus system.

The rate of leucine incorporation by rat liver particles was about 0.1 μmole/gm. protein/hour. Incorporation reached a maximum value in less than a half hour. No stimulation of incorporation was observed upon addition of an unlabeled amino acid mixture. The system studied by Siekevitz was composed of mitochondria and microsomes. Zamecnik and Keller found that incorporation proceeded under anaerobic conditions in a system composed of microsomes, a soluble non-dialyzable fraction, and a suitable energy source.

In the two bacterial systems leucine incorporation was much more rapid -- about 10 to 20 μmoles/gm. protein/hour. The Staph. aureus system was similar to that from liver since in the absence of an endogenous amino acid supply, maximum incorporation was attained in about an hour. In the presence of amino acids, incorporation increased linearly with time for at least 3 hours (longest period measured); an energy source was also required. Incorporation in M. lysodeikticus continued to increase for longer periods (up to 12 hours) in the absence of added amino acids although the net amount of incorporation was never much higher than that observed in Staph. aureus.

These differences among the three systems might be explained by considering these systems as representing different degrees of completeness in their protein synthesis mechanisms. The M. lysodeikticus system would thus be viewed as being most complete, having an endogenous energy supply, a limited amino acid supply, and synthesizing more protein upon addition of a complete amino acid mixture. The Staph. aureus system would be less complete, requiring an endogenous energy source and having the ability to synthesize new protein only in the presence of added amino acids. Least complete would be the rat liver system, which required an exogenous energy supply and was not stimulated by exogenous amino acids because possibly some unknown component of the complete system was absent or was damaged in the preparation. In this regard further fractionation of the M. lysodeikticus system seems pertinent.

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