Method of purification of DNA: Although we showed in the previous paper[1] that the hot phenol extraction leads to excellent results in the purification of DNA, it is obvious that the uncontrolled addition of too much phenol to a hot solution could lead to the denaturation of the DNA. To avoid this danger we have lately adopted the following protocol.

The DNA-containing material is suspended in a "1-molar" pH 7 buffer (NaCl, 1 *M*; EDTA, 0.001 *M*; NaH_2PO_4, 0.002 *M*; Na_2HPO_4, 0.006 *M*) and warmed to 55°C in a glass centrifuge tube in a water bath. An equal volume of buffer-saturated phenol is then added dropwise and the mixture allowed to stand at 55°C for about 5 min. At this temperature the phenol phase has practically the same density as the salt solution, so that phenol drops remain suspended throughout the solution and all parts of the solution come in close contact with the phenol without the need of shaking. The solution is then chilled and centrifuged cold to remove the suspension of fine reprecipitated phenol and denatured protein, if any. The phenol treatment may be repeated as many times as desired. Finally, the supernatant is transferred with a wide-mouthed pipette and the dissolved phenol is dialyzed away.

The solubility of phenol in the "1-molar" buffer at 60°C was found to be somewhat less than 6 per cent. The curves of the accompanying figure show that DNA is safely below the irreversible melting point at this temperature and phenol concentration at 0.195 *M* salt, and even more so at 55°C in the presence of 1 *M* salt, which latter has a stabilizing effect on DNA.[2, 3] Therefore, no critical control of the temperature or of the amount of phenol is required, and rough handling, e.g., shaking, is also unnecessary.

We are indebted to Mr. Andrew Smith and to Mr. Robert Cooper for technical help.

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*OVER-ALL SYNTHESIS OF ISOLEUCINE BY MEMBRANE FRACTIONS OF SALMONELLA TYPHIMURIUM**

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The properties of each of four enzymes—condensing enzyme, reductoisomerase, dihydroxy acid dehydratase, and transaminase—required for the conversion of pyruvate to valine or of pyruvate plus α-ketobutyrate to isoleucine have been characterized by a number of investigators for a variety of different organisms ranging from bacteria and fungi to higher plants. However, it has been demonstrated recently that these four enzymes are associated with the mitochondrial fractions of *Neurospora crassa;* these fractions contain a functional complex capable of converting substrate through the several enzymatic reactions to end product.[1-3]

Following this finding, it was of interest to investigate the possibility of subcellu-

lar organization of these enzymes in bacteria which have no discrete organelle comparable to the mitochondrion. The present report presents evidence that a membrane fraction of *Salmonella typhimurium* contains enzymes which carry out the over-all conversion of pyruvate plus α-ketobutyrate to isoleucine.

Materials and Methods.—Routinely, a broth culture of *Salmonella typhimurium*, wild-type strain LT-2, grown on a reciprocal shaker at 37°C for 8 hr, provided an inoculum for minimal medium[4] which was supplemented according to the method of Diena *et al.*[5] with a final concentration of 2 per cent glucose, 2 per cent glycine, and 0.01 *M* $MgSO_4$. Glycine in the medium induces spheroplast formation; glucose and $MgSO_4$ stabilize spheroplasts against osmotic lysis. Two liters of spheroplasts, which had been grown 15 hr at 37°C on a reciprocal shaker, were used for each experiment.

Spheroplasts were collected by centrifugation and washed once in cold 0.5 *M* sucrose containing 0.05 *M* Tris buffer at pH 8.5 by centrifuging at 5,000 × *g* for 10 min. They were then resuspended in the sucrose-Tris medium and subjected to sonication for 1 min (or in a few trials to sand grinding). The crude sonicate was washed by a fivefold dilution with sucrose-Tris before fractionation. Whole cells were removed from the crude sonicate by centrifuging at 5,000 × *g* for 10 min (supernatant designated S1). Next a membrane fraction was centrifuged down at 39,000 × *g* (resulting fractions designated P2 and supernatant S2) for 30 min which provided material for analysis of enzyme activity. Washed pellets (P3 and P4) were obtained by resuspending the P2 fraction in sucrose-Tris and centrifuging at 39,000 × *g*. All manipulations of the preparation were carried out at 0–4°C.

The synthesis of isoleucine from pyruvate plus α-ketobutyrate or intermediate substrates or valine synthesis from intermediates by *Salmonella* extracts was determined by the same methods as those employed for the measurement of a similar synthesis carried out by *Neurospora* extracts.[2] Procedures employed for the incubation of enzyme extracts with appropriate substrate and coenzyme concentrations were the same as those described previously.[2, 3] Routinely 2 mg of protein per ml of assay mixture were incubated at 37°C on a reciprocal shaker. Control incubations were complete assay mixtures except for the omission of substrate. The formation of isoleucine or valine was measured by assay with *Leuconostoc mesenteroides* P-60.[6]

α-Acetolactate, α-aceto-α-hydroxybutyrate, and α-keto-β-methylvalerate were synthesized in this laboratory by previously described methods.[9–11]

Protein was measured by the method of Lowry *et al.*[7] Dehydratase activity was measured according to the method outlined by Wagner *et al.*[8]

Results.—The synthesis of isoleucine from pyruvate plus α-ketobutyrate, or of valine from pyruvate, proceeds through a sequence of four enzymatic reactions as follows. (1) "Active" acetaldehyde reacts with pyruvate or α-ketobutyrate to produce α-acetolactate (AL) or α-aceto-α-hydroxybutyrate (AHB), respectively, in the presence of the condensing enzyme. (2) The α-aceto acids (AL and AHB) are converted in the presence of the reductoisomerase to the respective dihydroxy acids, α,β-dihydroxyisovalerate (DHV) and α,β-dihydroxy-β-methylvalerate (DHI). (3) The dihydroxy acids are dehydrated with the production of α-keto acid analogues of valine and isoleucine in the presence of the dehydratase. (4) The α-keto acids are aminated to valine and isoleucine in the presence of a donor

TABLE 1

ISOLEUCINE AND VALINE SYNTHESIS BY CELL FRACTIONS OF *Salmonella*

Substrate for isoleucine synthesis*	Sonication (1 min) Expt. I Pellet	Sonication (1 min) Expt. I Sup.	Sonication (1.5 min) Expt. II Pellet	Sonication (1.5 min) Expt. II Sup.
Pyruvate, αKB	0.221	0.00	0.061	0.016
AHB	0.104	0.61	0.026	0.778
DHI	0.474	1.91	0.513	2.65
KI	0.518	1.49	0.653	3.62
Valine synthesis*				
Pyruvate	0.00	0.00	0.056	0.00
AL	0.00	0.00	0.00	0.01
DHV	0.406	2.08	0.502	3.8
KV	0.521	1.22	0.806	2.75
Dehydrase activity keto acid synthesis†				
DHI	—	—	0.592	4.72
DHV	0.87	5.80	0.758	6.07

* μM Amino acid per mg protein per 3.5 hr.
† μM Keto acid per mg protein per 1 hr.

amino acid and the transaminase. These four steps have been shown to occur in over-all synthesis of valine and isoleucine from pyruvate and α-ketobutyrate in the presence of the mitochondrial fraction from *Neurospora* cells.[1-3]

Table 1, a summary of data from two separate experiments, illustrates the capacity of *Salmonella* membrane fractions and their supernatant fractions (see P2 and S2 in *Methods*) for synthesis of isoleucine or valine from each of their four substrates required in the over-all reaction. Isoleucine is synthesized by pellet fractions from any of the four substrates in appreciable amount, while supernatant fractions are unable to effect the enzymatic conversion of α-ketobutyrate to isoleucine. Valine synthesis from pyruvate was negligible in all experiments, although valine synthesis from intermediate substrates could be demonstrated. A comparison of isoleucine or valine amounts formed from the respective dihydroxy acids with the activity of the dehydratase enzyme alone shows that both pellet and supernatant fractions have much higher activity for converting the dihydroxy acid to keto acid than for synthesis of amino acid. This is to be expected since the rate of amino acid formation is dependent on the coordinate functioning of both the dehydratase and the transaminase. Supernatant fractions routinely show a higher specific activity than do pellet fractions for synthesis of both the amino acid and keto acid. However, the ratios of products from supernatant to those from their respective pellet fractions are similar for production of both amino acid and keto acid. The condensing enzyme has been measured and it was found that although supernatant fractions show a threefold increase in specific activity as compared to pellet fractions, only pellet fractions carry out the over-all synthesis of isoleucine.

Measurement of either individual enzyme activity or of amino acid synthesis from any of the last three substrates indicates that the supernatant fractions have increased activity for the individual enzymes over pellet fractions. Yet only pellet fractions synthesize isoleucine from α-ketobutyrate. Increasing the time of sonication of spheroplasts decreases pellet activity for isoleucine synthesis from either α-ketobutyrate or AHB. In other experiments membrane fractions were prepared from spheroplasts disrupted by sand grinding, a technique shown to preserve the biochemical integrity of mitochondria to a considerable extent. This technique also yields pellet fractions which retain the capacity to synthesize iso-

TABLE 2

EFFECT OF WASHING PELLET ON THE SYNTHESIS OF ISOLEUCINE FROM PYRUVATE PLUS α-KETOBUTYRATE OR FROM DHI

Wash medium	Fraction	μM Isoleucine/mg Protein/4 Hr for Substrates: Pyruvate + αKB	DHI
0.5 *M* Sucrose, 0.05 *M* Tris pH 8.5, 0.01 *M* Mg^{++}	S1	0.354	1.57
	S2	0.044	1.7
	P2	0.267 (85)*	1.4 (43)
	S3	0.049	2.7
	P3	0.218 (88)	0.42 (20)
	S4	†	†
	P4	0.159	0.118
0.5 *M* Sucrose, 0.05 *M* Tris pH 8.5	S1	0.357	1.54
	S2	0.043	1.62
	P2	0.289 (87)	1.03 (40)
	S3	0.032	2.84
	P3	0.241 (97)	0.34 (35)
	S4	0.011	0.518
	P4	0.181 (99)	0.077 (39)
0.05 *M* Tris pH 8.5, 0.01 *M* Mg^{++}	S1	0.381	1.74
	S2	0.034	1.74
	P2	0.307 (89)	1.06 (36)
	S3	0.038	2.08
	P3	0.180 (95)	0.139 (22)
	S4	0.021	0.787
	P4	0.088 (98)	0.043 (38)

Spheroplasts were disrupted by 1 min sonication in 0.5 *M* sucrose, 0.05 *M* Tris pH 8.5, and 0.01 *M* Mg^{++}; 39,000 × *g* pellets were washed in media indicated as described in *Methods*.
* Values in parentheses indicate % total activity in pellets compared to their supernatants.
† Proteins too low in supernatant for assay.

leucine from all substrates. These results suggest that isoleucine synthesis by the over-all reaction requires an enzyme complex which is organized through membrane association.

Data summarized in Table 2 illustrate the degree of stability in membrane fractions of isoleucine synthesis from either pyruvate plus α-ketobutyrate or from DHI. Membrane fractions from three separate preparations were washed in sucrose-Tris-Mg^{++}, sucrose-Tris, or Tris-Mg^{++}, respectively. With all washes, enzymes for the synthesis of isoluecine from DHI can be solubilized and are recovered with highest activity in the supernatant fractions. *In contrast, 85–99 per cent of the activity for over-all synthesis is retained with the membrane fraction.* Apparently, sucrose stabilizes the over-all activity since 45–50 per cent of the specific activity remains in membrane fractions if sucrose is present in the washing medium, while 23 per cent is recovered in the pellet fraction washed with Tris-Mg^{++}.

Following a short lag, the over-all synthesis of isoleucine by the 39,000 × *g* pellet is approximately linear with time for at least 4 hr (Fig. 1). A similar lag was noted for isoleucine synthesis by mitochondrial preparations from *Neurospora*.[1, 2]

The optimum concentration of α-ketobutyrate for the synthesis of isoleucine by membrane fractions was determined by varying the amount of α-ketobutyrate while holding pyruvate constant at 50 μM/ml; this concentration of pyruvate is not rate-limiting (unpublished results). Maximum synthesis of isoleucine is obtained with 25–30 μmoles/ml and decreases sharply at higher concentrations (Fig. 2). A similar pattern of the relationship of α-ketobutyrate concentration to synthesis of isoleucine through the over-all reaction was noted for mitochondrial preparations of *Neurospora*.[3]

The cofactor requirements for the over-all conversion of α-ketobutyrate to iso-

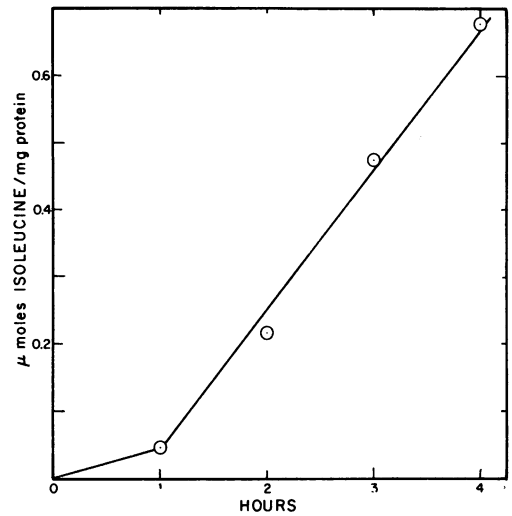

FIG. 1.—The production of isoleucine from pyruvate plus α-ketobutyrate in time.

leucine by 39,000 *g* pellet fractions are illustrated in Table 3. It is evident that omission of some of the cofactors essential for activity of the four separate reactions results in decreased over-all synthesis of isoleucine. The omission of TPP and Mg^{++}, however, has no or a slight effect on the production of isoleucine. Both of these are essential for the activity of the soluble condensing enzyme. It is also evident that the omission of pyruvate has a variable effect, but that in the absence of α-ketobutyrate no isoleucine production is detected. Isoleucine can also be synthesized from threonine, the precursor of α-ketobutyrate, in the presence of all cofactors and the membrane fraction; however, the specific activity is uniformly lower than that obtained when α-ketobutyrate is supplied as substrate, and in all cases there was no

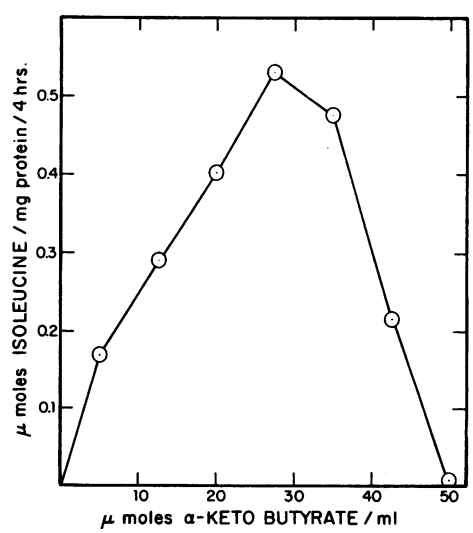

FIG. 2.—The effect of increasing the α-ketobutyrate concentration on the production of isoleucine. The level of pyruvate was held constant.

TABLE 3

REQUIREMENTS FOR THE OVER-ALL SYNTHESIS OF ISOLEUCINE BY 39,000 × *g* PELLET FRACTIONS FROM SPHEROPLASTS SONICATED 1 MIN

Omissions from complete assay	Expt. I	Expt. II	Expt. III	Expt. IV
0	100 (0.319)*	100 (0.284)*	100 (0.581)*	100 (0.632)*
αKB	0.0	2.4	—	2.2
Pyr	98.5	84.3	—	33.9
Pyr + αKB	0.0	0.0	0.0	0.0
All cofactors	7.5	13.3	2.9	2.9
B6′, φ ala	0.9	6.7	4.9	3.8
TPNH	37.3	33.3	44.7	30.2
TPP, Mg^{++}	97.2	113.2	86.5	94.5

Isoleucine synthesis is expressed as % activity of the complete system. The specific activity of each preparation is indicated.

αKB = α-ketobutyrate, pyr = pyruvate; B6′ = pyridoxal phosphate, φ ala = phenylalanine, TPP = thiamine pyrophosphate.

* μM Isoleucine/mg protein/4 hr.

stimulation of the over-all synthesis by the addition of pyruvate (unpublished results). This finding is not unique for *Salmonella;* Leavitt and Umbarger[12] detected an endogenous source of the 2-carbon donor while studying the condensing enzyme from extracts of *Escherichia coli.*

The pH optima for the over-all synthesis are depicted by the three experiments summarized in Figure 3. It is of special interest to note that a high specific activity for isoleucine synthesis occurs at two separate pH values. The optimum pH for maximum synthesis is apparently between 8.0 and 8.5, yet a second peak of activity exists between pH 6.5 and 7.0. This is not surprising if the pH optimum for the condensing enzyme is involved in the regulation of the over-all synthesis in bacteria. Condensing enzyme from *E. coli* extracts had been shown to exhibit two pH optima, 6.0 and 8.0.[13]

The effect of protein dilution on isoleucine synthesis from pyruvate and α-ketobutyrate and 39,000 × *g* pellet is shown in Figure 4. Isoleucine formation is proportional to amount of protein over a wide range of concentrations varying from 0.5 to 3.0 mg/ml of the assay mixture. Also illustrated is the fact that the specific activity for isoleucine synthesis remains constant over the entire range of protein concentrations tested, suggesting that the four enzymes function as a single unit which is not altered by protein dilution.

Discussion.—The data presented in this report provide evidence that isoleucine can be synthesized from α-ketobutyrate in the presence of appropriate cofactors and a particulate fraction from *Salmonella* spheroplasts which pellets at 39,000 × *g*. If membrane fragments are prepared by a treatment of either sonication for 1 min or sand grinding, the particulate fraction is active for the over-all synthesis, as well as synthesis of isoleucine from intermediate substrates. Neither treatment produces 39,000 × *g* supernatant fractions which carry out the over-all reaction, although these fractions contain higher activity than do pellet fractions for either individual enzymes or for isoleucine synthesis from DHI or α-keto-β-methylvalerate.

The various studies of properties of the over-all reaction suggest that isoleucine synthesis results from a coordinate functioning of the four enzymes involved. The true characteristics of this isoleucine-synthesizing system of necessity await further investigation. However, it is suggested on the basis of this preliminary study that isoleucine synthesis is made possible through ordered arrangement of the enzymes in a complex which is stabilized by membrane association. This would render the

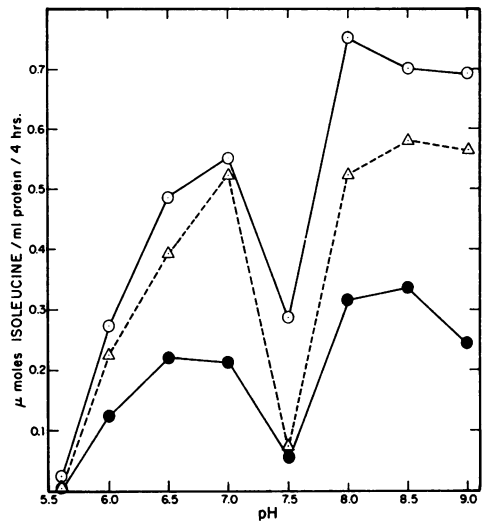

FIG. 3.—The effect of pH on the production of isoleucine.

situation in *Salmonella* comparable to that found in *Neurospora*,[1-3] where functional organization of these four enzymes is apparently dependent on mitochondrial membranes (unpublished results).

The inability of the membrane fraction to produce valine consistently from pyruvate is presently not understood, but it is evident that the results reported herein provide the framework for the study of the indicated subcellular organization of the multienzyme system for isoleucine synthesis in bacteria. Bacterial membranes have already been demonstrated to participate in biosynthetic reactions in that the electron transport system is localized in association with these structures.[14] Also, DNA-like RNA of *Escherichia coli* has been shown to be associated with the bacterial membrane.[15, 16]

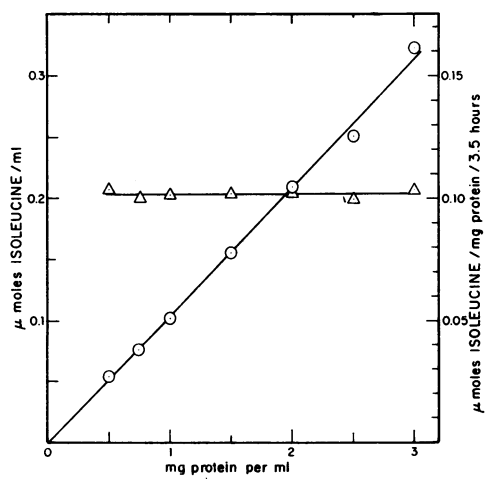

FIG. 4.—The effect of dilution on isoleucine production. —⊙—, Total μmoles of isoleucine synthesized per ml in 3.5 hr in presence of the indicated amount of protein. —△—, The specific activity for isoleucine synthesis at the indicated levels of protein concentration.

If membranes function in organizing gene products, either passively by providing a framework for stabilizing macromolecular complexes or actively, and if the lipoprotein moiety of the membrane acts in both structural and catalytic capacities, then studies of the over-all synthesis of isoleucine in *Salmonella* could be utilized to elucidate the relationship between closely linked genes and their products, and to test the possibility that a colinearity exists between genes, gene products, and organization of products in the cytoplasm in bacteria.

Summary.—The over-all synthesis of isoleucine from pyruvate and α-ketobutyrate is shown to occur in the presence of the membrane fraction of disrupted cells of *Salmonella typhimurium*, and not in the supernatant soluble fraction.

We wish to thank Dr. Frank B. Armstrong of the Department of Genetics of North Carolina State College at Raleigh for supplying the strain of *Salmonella* used in this study.

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THE ISOLATION AND SOME PROPERTIES OF RAT LIVER MITOCHONDRIAL DEOXYRIBONUCLEIC ACID

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Although the presence of DNA in rat and mouse liver mitochondrial fractions was noted a number of years ago,[1-3] DNA was not considered to be a true constituent of mitochondria because the presence of nuclear fragments in the isolated mitochondrial fractions could not be excluded and because the analytical techniques