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Regulation Of Protein Synthesis By Leucine And Amino Acid Balance.

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REGULATION OF PROTEIN SYNTHESIS BY LEUCINE AND AMINO ACID BALANCE

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Chairman

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ABSTRACT

The effects of a physiologically balanced mixture of amino acids on the synthesis of proteins has been investigated. The roles of leucine and tryptophan, both implicated in the regulation of protein synthesis, were also studied.

The balance of amino acids is important under specific rate-limiting conditions; the physiological balance "protects" the protein synthesizing system from the stressed condition of leucine limitation. Leucine is an important regulator of protein synthesis and the tryptophan effect on translation is dependent on the concentration of leucine. Thus tryptophan is a secondary regulator. The relative concentration of amino acids, described as balance, altered the synthesis of protein in cell-free and intact cell culture experiments, when leucine was limiting. Both qualitative and quantitative differences were observed. The effect of the amino acid mixture decreased when the concentration of leucine was physiological. Two different components were sensitive to added leucine. This sensitivity was indicated by different kinetics; one component showed low Km and Vmax values while the other showed high Km and Vmax values. The initial rate of protein synthesis was first order with respect to leucine when it was limiting and mixed order when it was physiological.

The effect of tryptophan on stimulation of protein synthesis was small in comparison to the effect of leucine, and was dependent on the concentration of leucine. The incorporation of leucine into protein was changed as the tryptophan concentration changed when leucine was limiting; synthesis of albumin was slightly stimulated. The ribosome distribution did not change as indicated by polysome analysis. The incorporation of leucine into protein did not change when leucine was physiological and tryptophan was varied. However, the ribosome distribution was altered.

A low molecular weight inhibitor of protein synthesis was found in cell extracts which acted independently of amino acid or leucine concentrations. It could be partially removed by treatment with G-25 Sephadex, but has not been purified.

Several nucleotide effects independent of amino acid concentration were also observed. ATP, at increasing concentrations, significantly depressed levels of synthesis and concentrations greater than 4 mM caused 100% inhibition of the protein synthesizing system. The phosphodiesterase inhibitor, theophylline, enhanced synthesis of albumin, although the cyclic nucleotide, cAMP, did not itself alter synthesis of protein.

Finally, the concentrations of amino acids in plasma of C^3HeB/FeJ mice were determined. The two amino acids examined in the protein synthesis experiments, tryptophan and leucine, were found to remain relatively constant, regardless of the fed or fasted condition of the animals, but showed changes with the age of the animals.

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INTRODUCTION

In recent years many investigators have turned their attention to the regulation of protein synthesis in eukaryotic systems. Analysis of the various stages of protein synthesis indicated that there were numerous reactions where possible control mechanisms could exist. However, these stages were studied independently of each other. It is not known whether the results obtained in this manner are relevant to the role that each of these specific stages would play in the intact organism.

The initiation stage, responsible for activation of the mRNA-ribosome complex, represents an excellent example. There are at least seven different protein factors involved in the formation of this complex (1-5). The functional significance of these proteins for the overall initiation process has been demonstrated, but in most cases their exact roles have yet to be determined (6). Several of these factors have been shown to bind to mRNA independent of ribosomes (7). However, this capacity for binding to mRNA appears to be relevant only under specific *in vitro* conditions, and is thought not to be relevant *in vivo* (8).

At the time this project began, few investigators addressed themselves to the question of physiological relevance; most experiments were designed to evaluate only a specific reaction of protein synthesis. Highly purified protein factors, mRNA, and ribosomes were prepared free of the membrane fraction. However, Pitot, et al. (9) reported that ribosome-membrane interactions were essential for studies of eukaryotic protein synthesis. This finding was confirmed by Hill, et al. (10) who demonstrated that *in vitro* patterns of proteins, as revealed by gel

electrophoresis were similar to those obtained *in vivo*, only if the integrity of the membranes was preserved, and the supernatant was not diluted. In addition, the importance of the 5' terminal caps of mRNA in protein synthesis was incorrectly described because the heterologous wheat germ and reticulocyte protein synthesizing systems were employed to evaluate cap function; the ionic condition under which the translational assay was performed influenced the apparent cap requirement *in vitro*, whereas in homologous systems, the presence of the cap was absolutely required (11).

Finally, it was reported that leucine inhibited protein synthesis *in vitro* at a concentration far removed from values reported to be present physiologically (12,13).

This investigation began as an attempt to define the molecular mechanism whereby Growth Control Factor (GCF) (14,15,16) relieved puromycin inhibition in intact cell culture studies and liver perfusion studies. When the liver tissue was disrupted, the GCF effect disappeared but ribosome binding occurred (Sayre, F.W., unpublished). This observation, and those referred to above, altered the direction of this research project; it was concluded that the GCF protein effect would not be observed until the translational parameters for liver protein synthesis were more carefully evaluated.

In order to study protein synthesis, investigators have utilized two types of translational systems: homologous systems in which all of the components are derived from the same tissue source, and heterologous systems in which a wheat germ S·30 fraction, or a reticulocyte lysate is used with mRNA or another component from an entirely different tissue or organism. The homologous system is advantageous for the study of

transcriptional products in a more physiological environment; the disadvantage is that the system is extremely complex. The heterologous system is advantageous for the study of the relationship between a specific mRNA and its translational product. It has provided much of our knowledge of the detailed mechanisms of initiation, elongation, and termination. The disadvantage of this system is that it is quite unphysiological; this system has provided artifactual results related to regulation of protein synthesis. For example, the requirement for specific initiation factors cannot be demonstrated in this system, but are necessary for synthesis in the homologous system (17).

A less fractionated, more highly intact, homologous protein synthesizing system would provide a closer correlation to the in vivo condition (10). Liver was chosen as the tissue of study. The major cells are hepatocytes which synthesize characteristic translational products that would be excellent markers for protein synthesis, e.g., albumin and transferrin (18-21).

Finally, a specific strain of mice, C3HeB/FeJ was chosen for these studies. This strain was well established and had been used for various liver studies (22). The male of this species has a 90% incidence of spontaneous hepatocarcinoma at approximately 21 months of age. Since in other studies of the protein, GCF was shown to affect tumor growth, this represented a strain in which spontaneous liver tumor formation in the older animal could be studied (Sayre, F.N., unpublished). Protein synthesis and GCF effects could be characterized for this system. The tumor studies could be the subject of future research investigations.

It became apparent that one parameter which varied extensively and rather haphazardly, in cell-free protein synthesis assays, was the supply

of amino acids. Most studies failed to take into account the relative concentrations of amino acids under normal conditions. Some standard published procedures call for amino acid concentrations based on the relative composition of hydrolyzed casein or other naturally occurring specific proteins, but most have relied on the use of equimolar mixtures of amino acids as starting points for protein synthesis (23). A few other studies have used amino acids which are present in equal weight concentrations; these have been reported to produce faster rates of protein synthesis than did equimolar mixtures (24).

The synthesis of any protein requires the simultaneous presence of each constituent amino acid in sufficient amount to meet the needs for the protein being synthesized. An excess of any individual amino acid cannot lead to synthesis of greater amounts of protein because other amino acids would become limiting with regard to the amount of protein synthesized. There is no direct mechanism for the storage of amino acids per se, and all of the protein synthesizing machinery must obtain its supply of amino acids from the amino acid pool. Intracellular pools can be depleted by the synthesis of proteins. These intracellular pools can also be replenished by supply from the peripheral circulation. At any particular time, the synthesis of an individual protein will be limited by availability of the amino acid required in greatest amount and in least supply. All other amino acids will be present in proportionate excess. The major supply of amino acids will come from dietary intake. The diet should include all amino acids in sufficient supply to meet the needs of the cell. The major portion of these needs will be for protein synthesis.

Many studies have shown that the plasma concentrations of amino acids remain constant within relatively narrow limits (25). The plasma pool of amino acids represents the major supply of all tissues. It would seem then that these plasma concentrations would represent adequate amounts of each individual amino acid for the purposes of the cell. The synthesis of proteins under physiological conditions, then, would have these concentrations supplied to the tissues. We have referred to these relative concentrations as found in plasma, as the plasma balance of amino acids.

Previous nutritional studies on cultured tissue cells (26-28) and multicelled organisms (nematodes in axenic culture) (29) have established very clearly the importance of the balance of amino acids supplied to the growing system. Because of these findings, it seemed important to investigate the influence of the balance of amino acids upon protein synthesis.

The objectives of this investigation were to evaluate certain translational parameters of the liver cell-free system with respect to their physiological significance.

The first objective was to determine the importance of the relative concentration of amino acids, termed balance, on the extent of protein synthesis. The second objective was to determine the effect of leucine on protein synthesis. The observed inhibition of protein synthesis in rat liver mitochondria (12), a report of leucine inhibition of glycyl-tRNA synthetase in rat liver (13), and the observed inhibition of mouse liver protein synthesis (Fig. 8), imply that leucine may be extremely important as a regulator of protein synthesis. In addition, leucine had been shown to enhance the mitogen induced stimulation of lymphocytes

in cell culture (30). A third objective of this investigation was related to the role of tryptophan in protein synthesis. Several studies have demonstrated that tryptophan affects the distribution of hepatic polyribosomes (31-33), and of mRNA specific for albumin (34,35).

Since the amino acid values present in the literature were for mice in general, and no strain designation was stated, the fourth, and final objective was to obtain the plasma values of amino acids for the C3HeB/FeJ strain used in this investigation.

This investigation was intended to provide knowledge of several parameters involved in protein synthesis in relation to physiological conditions.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from Sigma Chemical Co., Saint Louis, Mo.: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), 2-amino-2-hydroxymethyl-1,3,propanediol (Tris-Cl), phospho(enol) pyruvate (PEP), ATP: pyruvate 2-O-phosphotransferase (pyruvate kinase, PK), magnesium chloride ($MgCl_2$), potassium chloride (KCl), bovine serum albumin (BSA) and all amino acids.

Coomassie Brilliant Blue (R250), agarose (HGT), mouse albumin (Cohn Fraction V), rabbit anti-mouse albumin serum, and goat anti-rabbit serum were obtained from Miles Research Products Div., Elkhardt, In. N,N'-Methylene-bis-acrylamide (Bis), riboflavin, acrylamide, ammonium persulfate, sodium azide, Biophore 7.5% gels, sodium diethybarbiturate, and diethylbarbituric acid were obtained from Bio-Rad Laboratories, Richmond, CA.

For amino acid analysis ninhydrin, sodium acetate (4M), titanous chloride, lithium hydroxide (LiOH), lithium citrate (pHix Pico Buffer System IV), 5-sulfosalicyclic acid, ninhydrin spray, Piersolve (2-methoxy ethanol), and Amino Acid Standard Physiological ANB were obtained from Pierce Chemical Co., Rockford, IL.

Norleucine standard, 2.5 μ mole/ml, was obtained from Technicon Corp., Chauncey, New York.

Sephadex G-25, medium, and Blue Dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Toluene, 2,5-diphenyloxazole (PPO), 1,4-bis [2-(5-phenyl-oxazolyl)]-benzene (POPOP), and Bio-Solv (BBS-3), were purchased from Beckman Inst., Palo Alto, CA.

NCS tissue solubilizer was obtained from Amersham/Searle Corp., Arlington Heights, IL.

N,N,N',N'-tetramethylethylenediamine (TEMED) was obtained from Eastman Kodak, Rochester, New York.

Dulbecco Modified Eagles medium (DME) and 10% heat inactivated dialyzed calf serum were obtained from Grand Island Biological Co., Grand Island, New York.

Finally, 2-mercaptoethanol was obtained from Cal-Biochem, San Diego, CA.

Animals

Inbred male mice of the strain C3HeB/FeJ used in these experiments were obtained from Jackson Laboratories, Bar Harbor, Maine. These were maintained in a constant temperature room at 22°C with automatic day-night light cycles of twelve hours light and 12 hours dark. Tap water

and a laboratory diet in pellet form (Simonson Lab., Gilroy, CA.) were given ad libitum. The weights of the animals were recorded for each experiment. All animals were within 2.5 - 3.5 months of age (mean life span, 21.9 months) and weighed 29.6 ± 5.4 g when sacrificed.

Preparation of Supernatant Fractions for In Vitro Experiments

Fed or fasted conditions were established for the animals either by maintenance of the food supply or by removal of food pellets 12 hours prior to sacrifice. Plasma levels of the amino acids were thus established for the starved or fed condition. The fractionation technique of Hill, et al (10) was modified in the following manner:

Animals were killed by cervical fracture. The livers were removed from these animals, pooled, washed in Medium B: 250 mM sucrose, 25 mM KCl, 8 mM Mg Cl_2, 5 mM 2-mercaptoethanol and 100 mM Tris-Cl [2-Amino-2-hydroxymethyl-1,3 propanediol] (pH 7.4 @ 4^oC), and weighed. Subsequent procedures were performed at 0^o to 4^o C. Tissues were minced and homogenized in two volumes of Medium B (w/v) in a Thomas type C homogenizer for 1 min. The 33% homogenate was centrifuged at 10,000 x g_{max} for 10 min (Sorvall RC-2, Type ss-34 rotor). The resulting supernatant, designated the 10 K-S (10,000 x g_{max} supernatant), excluding the upper lipid layer, was removed and stored in small aliquots at -76^oC until further use in the protein synthesis assay.

For specific experiments, the 10 K-S fraction was passed through a Sephadex G-25 chromatographic column (24 cm x 1.8 cm, Vo = 13 ml, V_+ = 38 ml). The eluate, collected at the void volume of the column, was then used in the assay. This fraction was designated 10 K-S(G-25).

A more fractionated system was derived from the 10,000 x g_{max} treated supernatant. This fraction was centrifuged at 40,000 r.p.m. for 2.5 hrs (Beckman L3-40, Type 65 rotor). The resulting supernatant, designated 40 K-S, excluding the upper 1/3, which contained a lipid layer, was stored at -76°C for later use in protein synthesis. The precipitate, which is the microsome fraction, was gently resuspended in Medium B by manual homogenization in a Thomas Type B homogenizer. The suspension, designated 40 K-M, was then stored in small aliquots at -76°C for use in protein synthesis.

Samples were not thawed and refrozen since an appreciable loss in activity (approx. 15%) was noted after a single freeze and overnight thaw. However, this loss did not alter the amino acid effect on protein synthesis. No additional loss of activity occurred with these stored fractions over a period of 6 to 9 months.

Preparation of Supernatant Fractions for In Vivo Experiments

Mice were starved for a period of 12 hrs before sacrifice for fasting experiments. Mice were injected intraperitoneally 10 minutes before sacrifice with either a ^{14}C-labelled amino acid mixture or a ^{3}H-labelled amino acid mixture. Each mixture contained 32 µmoles of each amino acid after final adjustment of labelled and unlabelled amino acids. Only leucine, lysine, isoleucine, and phenylalanine were radio-labelled. These four amino acids were chosen because dilution in a precursor pool, or degradation and utilization of these compounds as metabolites for alternate pathways is limited in comparison to the other amino acids. For the L-[µ^{14}-C] amino acid mixture, 10 µ Ci of each of the following were added: L-[µ^{14}-C] leucine (specific activity,

342 mCi/mmole), L-[^{14}-C] iso-leucine (specific activity, 342 mCi/mmole), L-[μ^{14}-C] lysine (specific activity, 345 mCi/mmole), and L-[μ^{14}-C] phenylalanine (specific activity 522 mCi/mmole). The injection volume was adjusted with physiological saline to 80 µl.

The L-[^{3}H] amino acid mixture contained 40 µCi of each of the following: [L- 4,5-^{3}H] leucine (specific activity, 30 Ci/mmole), L-[4,5-^{3}H] isoleucine (specific activity, 30 Ci/mmole), L- 4.5-^{3}H lysine (specific activity, 30 Ci/mmole), and L-[3-^{3}H, ring - 4-^{3}H] phenylalanine (specific activity, 6.6 Ci/mmole). The injected ^{3}H/^{14}C ratio was established as 4:1. The injection volume was adjusted with physiological saline to 80 µl.

The animals were terminated by cervical fracture 10 min after injection and the livers were excised and weighed. An equal amount of tissue from ^{14}C- and ^{3}H-treated animals was pooled, chilled, minced, and homogenized in 2 volumes (w/v) of Medium A: 250 mM sucrose, 25 mM KCl, 8 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.0 at 37^{o} C. The homogenate was centrifuged at 15,000 x g_{max} (Sorvall RC-2, SS-34 rotor) for 10 min. The upper 2/3 of the supernatant, excluding the lipid layer was removed and centrifuged at 139,000 x g_{max} (Beckman L3-40, Type 65 rotor) for 2.5 hrs. The resulting microsomal pellet was sonically disrupted with a Brinkman Polytron (60 second, dial setting 5) and recentrifuged at 139,000 x g_{max} (Beckman L3-40, Type 65 rotor) for 2 hrs. The protein concentration of the supernatant was determined and the supernatant was stored at -76^{o}C prior to electrophoresis.

Preparation of Amino Acid Mixtures for Protein Synthesis

Mixtures of amino acids were prepared according to the designed

balance using commercial preparations of L-amino acids of the highest purity available. Individual amino acids were authenticated for identity and purity by two dimensional chromatography on cellulose thin-layer plates. Amino acid standards were obtained from Sigma Chemicals, St. Louis, MO. Development in the first dimension was carried out with Solvent I; n-Butanol-acetic acid-water (4:1:5). The plates were then dried for 5 - 10 min at 30°C and subsequently developed in the second dimension with Solvent II: pyridine:H_2O (4:1). The sheet was allowed to dry and then sprayed with ninhydrin for the detection of amino acids. Stock solutions of amino acids were prepared at appropriate concentrations (i.e., 10-fold, 100-fold, etc.) and stored in small aliquots in sealed tubes at -76°C.

A. Plasma balanced mixtures. The term balance, as used in these experiments, refers to the relative concentrations of the amino acids. This amino acid mixture was designed according to the literature values for plasma concentrations of amino acid in "mice" (36); species and strain designations were not specified. In addition, several amino acid values were not reported in the literature, i.e., those for serine, and aspartic acid. These values were obtained by an evaluation of the data available for other rodents. The formulation is shown in Table 1. It was recognized that the available values were not all of the same reliability, and that they were not obtained with the specific fasting and fed plasma levels of amino acids for C3HeB/FeJ. It was ultimately necessary to determine the specific fasting and fed plasma levels of amino acids for C3HeB/FeJ. This is described in detail in a subsequent section.

Table 1

Amino Acid Compositions

Amino Acid "ESSENTIAL"	Balanced Mixture (Plasma Values) mg/100 ml	mM[1]	Equimolar Mixture[2] mg/100 ml	DME[3] mg/100 ml	mM	Ratio[4] Balanced:Equimolar mM / mM ARG	Ratio[5] Balanced:DME mM / mM ARG
ARGININE	0.97	0.058	3.81	8.4	0.502	1.00	1.0
HISTIDINE	1.62	0.104	3.40	4.2	0.269	1.79	3.34
ISOLEUCINE	1.52	0.116	2.87	10.5	0.801	2.00	1.12
LEUCINE	2.40	0.183	2.87	10.5	0.800	3.15	1.25
LYSINE	6.57	0.360	4.00	14.6	0.800	6.21	3.89
METHIONINE	1.96	0.132	3.26	3.0	0.202	2.28	5.66
PHENYLALANINE	2.47	0.150	3.61	6.6	0.400	2.56	3.21
THREONINE	3.61	0.303	2.61	9.5	0.797	5.22	3.29
TRYPTOPHAN	1.27	0.062	4.47	1.6	0.078	1.07	6.88
VALINE	4.43	0.378	2.56	9.4	0.802	6.52	4.08
"NONESSENTIAL"							
ALANINE	5.87	0.670	1.95	-	-	11.55	-
ASPARAGINE	1.72	0.115	2.89	-	-	1.98	-
ASPARTIC	0.20	0.015	2.92	-	-	0.26	-
CYSTEINE	1.07	0.061	3.83	-	-	1.05	-
GLUTAMIC	3.43	0.243	3.22	-	-	4.19	-
GLUTAMINE	9.20	0.630	3.20	-	-	10.86	-
GLYCINE	1.96	0.261	1.64	3.0	0.399	4.50	5.66
PROLINE	1.89	0.164	2.52	-	-	2.83	-
SERINE	1.93	0.184	2.30	4.2	0.400	3.17	3.97
TYROSINE	2.61	0.140	3.97	7.2	0.386	2.41	3.13
CYSTINE	-	-	-	4.8	0.273	-	3.86
		Σ = 4.3 mM[1]	Σ = 4.3 mM[2]			Ave. = 0.749 mM ± 0.258 mM for the 14 amino acids present	
	Ave. = 0.216 ± .178 mM		Ave. = 0.218 mM				

(continued - Footnotes on following page)

Table 1 (continued)

Footnotes

[1]Composition was based on average plasma values reported for Mus musculus (36). In those specific cases where an amino acid value had not been determined the average plasma value was obtained by a statistical analysis of given mammalian plasma values (Asn, Asp, Cys, Gln, Ser).

[2]Each amino acid was present at a concentration of 0.218 mM. The sum total amino acid concentration was 4.3 mM which corresponds to the value obtained for balanced plasma values.

[3]Dubeecco's Modification of Eagle's Minimum Essential Medium Values were obtained from Dulbecco, et al (40).

[4]The ratio of balanced/equimolar amino acid mixture was obtained by arbitrarily establishing the concentration of Arg as 1.00 mM and determining the mM concentration of each amino acid relative to this. The ratio of balanced/equimolar was the value obtained by dividing mM/mM Arg for the specific amino acid in the balanced mixture by the mM/mM Arg value in the equimolar mixture. This normalized all values and established a reference ratio for comparison with other experiments.

[5]The same procedure as in footnote 4 was applied to Dulbecco's Modification of Eagle's Minimum Essential medium.

B. Cell culture balanced mixture. The amino acid balanced culture media was made from Dulbecco's modified Eagle's medium (DME) prepared according to the published formulation (37), except that all amino acids were omitted. Molar concentrations of the amino acids in Dulbecco's medium vary from 0.078 mM tryptophan, to 0.800 mM lysine, to 3.99 mM glutamine. An amino acid supplement, prepared at forty-fold concentration and sterilized by filtration, was used to adjust the amino acid levels of compounded media to the desired strength and composition (Table 1). The normal concentration of arginine in DME is 0.400 mM. Arginine is an absolute requirement for growth and survival of 3T3 mouse and other fibroblast cell lines, and has been chosen as a reference for design of balanced amino acid mixtures (38). A reference equimolar mixture was tested with each amino acid present at 0.400 mM concentration. The experimentally balanced media was tested against this reference equimolar mixture and against the regular Dulbecco's medium with regard to its ability to support growth and rate of protein synthesis. Special attention was paid to total amino acid concentration, as well as to the concentration of individual specific amino acids in the comparative study.

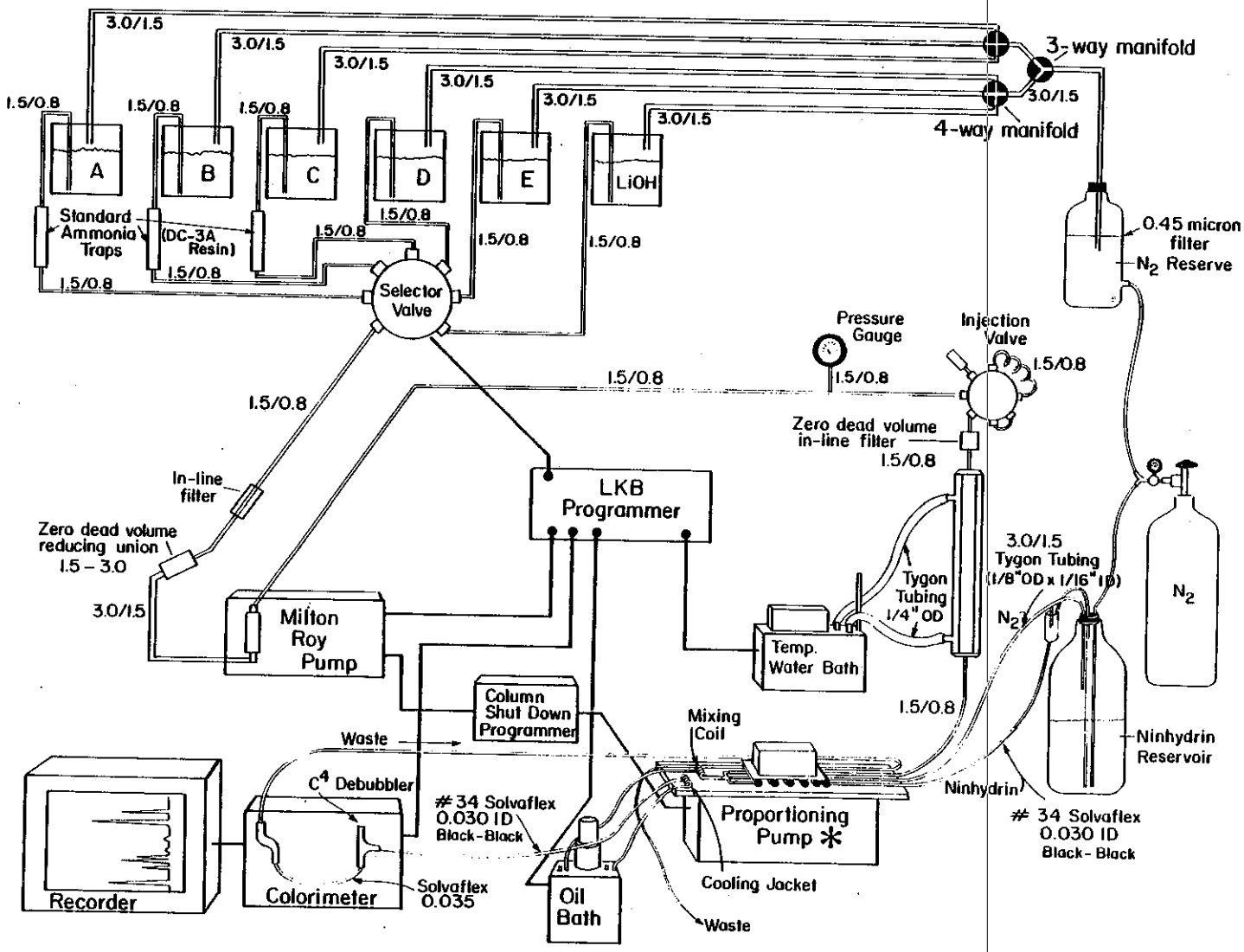
Amino Acid Analysis of Plasma fr C3 HeB/FeJ

A. Sample preparation. Whole blood was obtained from mice by the tail bleed method. The tail of the restrained animal was carefully shaved and cleaned. The caudal vein was lanced and blood collected in Natelson heparinized capillary tubes (250 µl volume). The blood was transferred to polystyrene centrifuge tubes and centrifuged immediately at 5,000 x g for 2 minutes in a Fisher Model 59 microcentrifuge (Fisher Sci., Santa Clara, CA). The plasma was removed and transferred to

another centrifuge tube, and 10 µl of 50% sulfosalicylic acid were added per 100 µl of plasma. The mixture was gently mixed for 5 minutes, then centrifuged at 5,000 x g for 15 minutes as stated above. The supernatant was decanted and adjusted to a pH of 2.2 with 4% LiOH. The solution was recentrifuged at 7,000 x g for 20 min. The supernatant was withdrawn and frozen at -20°C until amino acid analysis.

B. Single column method for amino acid analysis of physiological fluid. A Technicon Auto Analyzer (Technicon Corp., Chauncey, NY) was modified for the single column method of separation (Fig. 1). A jacketed column containing Durrum DC-6A cation exchange resin was used for the separation (Durrum Chemical Corp., Sunnyvale, CA). The flow rate was maintained by means of a Milton Roy high pressure positive displacement pump (Laboratory Data Control Corp., State College, PA). The maximum eluent pressure did not exceed 550 psi. The column outlet was connected to a Technicon Auto Analyzer system which controlled the mixing of the eluted amino acid(s) with the ninhydrin reagent (Fig. 2). The amino acid-ninhydrin mixture was passed through a 45 ft., 3 mm I.D. glass reaction coil maintained at 95°C in a temperature-controlled oil bath. Dwell time in the reaction coil was approximately 10 min. Transmittance of the "Ruhemann's Purple" complex was measured by a Technicon Colorimeter (Technicon Corp., Chauncey, NY) at 570 nanometers, and the signal was recorded on a Technicon multiple-point recorder. The physical parameters for biological fluid analysis are shown in Table 2.

A commercially formulated Lithium Pico-Buffer system (pHix Lithium Pico-Buffer System IV, Pierce Chemical Co., Rockford, IL) was used to separate and elute the amino acids. The five buffer solutions plus the regenerating solution (0.3 M LiOH) were filtered through a 0.5 µm pore


3.0/1.5
3-way manifold
4-way manifold
1.5/0.8
A
B
C
D
E
LiOH
Standard Ammonia Traps
(DC-3A Resin)
Selector Valve
0.45 micron filter
N_2 Reserve
Pressure Gauge
Injection Valve
Zero dead volume in-line filter
In-line filter
Zero dead volume reducing union 1.5 – 3.0
LKB Programmer
Milton Roy Pump
Temp. Water Bath
Tygon Tubing 1/4" OD
3.0/1.5 Tygon Tubing (1/8"OD x 1/16"ID)
Column Shut Down Programmer
Mixing Coil
Waste
C^4 Debubbler
#34 Solvaflex 0.030 ID Black-Black
Proportioning Pump *
Ninhydrin Reservoir
Ninhydrin
Recorder
Colorimeter
Solvaflex 0.035
Oil Bath
Cooling Jacket

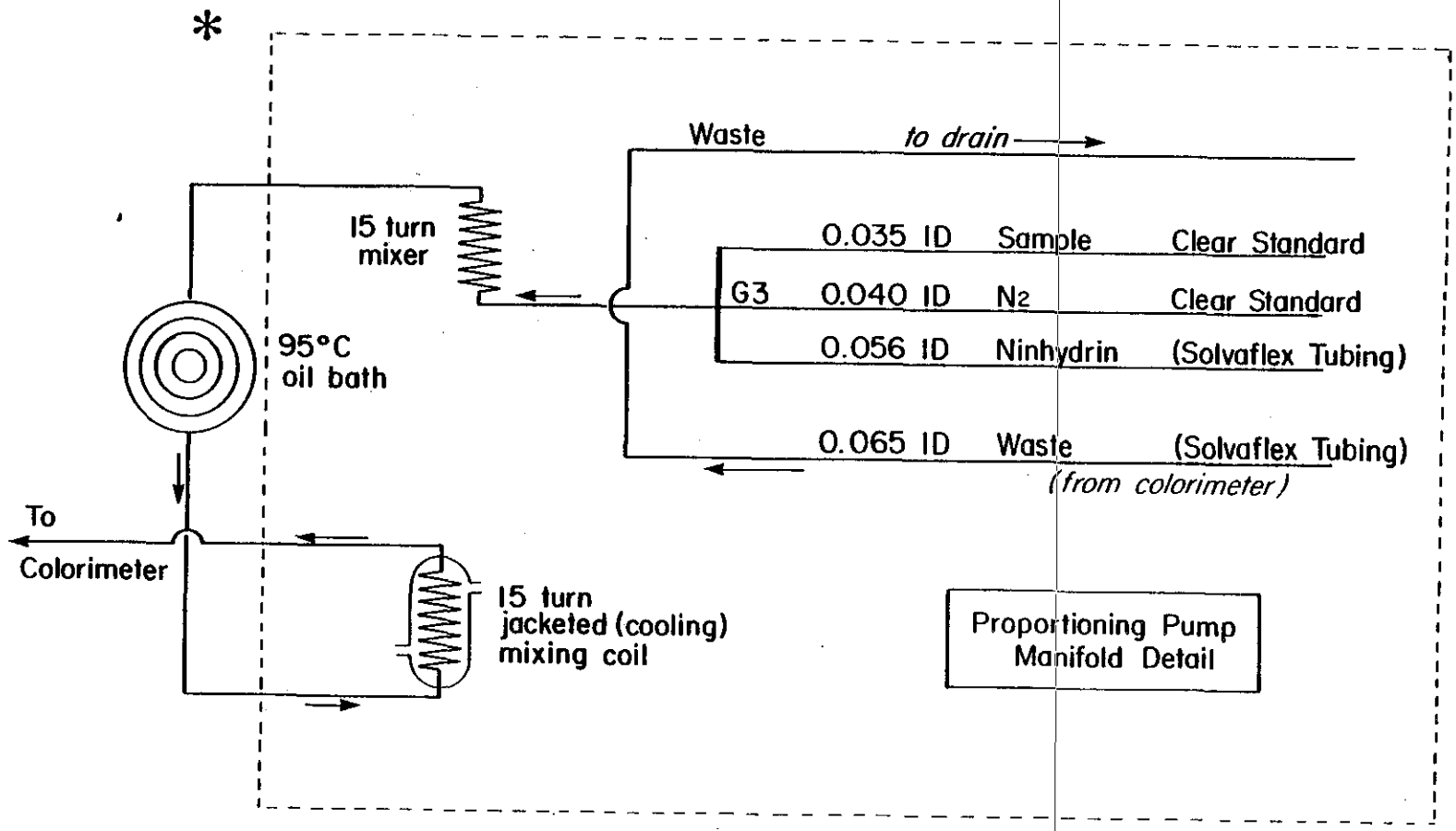

*
Waste
to drain
15 turn
mixer
0.035 ID
Sample
Clear Standard
G3
0.040 ID
N2
Clear Standard
0.056 ID
Ninhydrin
(Solvaflex Tubing)
95°C
oil bath
0.065 ID
Waste
(Solvaflex Tubing)
(from colorimeter)
To
Colorimeter
15 turn
jacketed (cooling)
mixing coil
Proportioning Pump
Manifold Detail

Table 2

Parameters for Amino Acid Analysis
of Biological Fluids

Bed Dimension (cm)	0.9 x 32
Resin (Dionex Corp., Sunnyvale, CA.)	DC - 6A
Eluent Flowrate ($ml \cdot h^{-i}$)	30
Maximum Pump Pressure (psi/atm)	550/38
Column Temperature, T_1 (^oC)	36
Column Temperature, T_2 (^oC)	60
Temperature Change Time (min)	100
Proportioning Pump Speed ($ml \cdot h^{-i}$)	30
Reaction Coil Internal Diameter (mm)	2.0
Reaction Coil Dwell Time (min)	10
Reaction Coil Temperature Bath (^oC)	95
Flow Cell Dimension (mm)	15
Detector Wavelength (nm)	570

filter disk prior to entering the column. In addition, the first three buffers were each passed through individual cationic exchange resins (DC-3 resin, Durrum Corp., Sunnyvale, CA) to remove ammonia. Ammonia interferes with detection of the amino acids and causes excessive base-line shifts. The six position eluent selector valve (Glenco SSV6, Glenco Corp., Houston, TX) was interfaced to an LKB 4930 programmer (LKB Corp., Stockholm, Sweden) which controlled the buffer selection time. The buffer parameters are listed in Table 3. Ninhydrin reagent was prepared by the method of Spackman, et al (39) and stored under nitrogen at room temperature. Prolonged storage was at $4^{o}C$.

C. Water purity. Conventional methods for water purification proved to be inadequate with respect to obtaining water suitable for buffer preparation. Twice glass distilled water was put through a pre-filter and a 0.45 micron filter (Millipore Corp., Bedford, Mass). This water was then redistilled in a double still containing quartz elements. The water was collected in liter bottles and stored under ultra high purity nitrogen gas (Hickinbothom Bros., Ltd., Stockton, CA). Water purity was monitored by a conductivity bridge. The most acceptable water had a purity greater than 18 megohms cm^{-1}. Ammonia and free amine content were monitored by the use of Nessler's Reagent. This was necessary since the system was sensitive (in the picomole range) to free amine and ammonia levels.

D. Calibration of column and detector response. Elution data was calibrated by analysis of standard amino acid mixtures. Initial mixtures contained amino acids whose elution time and position differed considerably from each other. Other amino acids were subsequently added to this

Table 3

Buffer System for Biological Fluid Analysis

Pico Buffers	pH	Begins prior to	Elutes through	Time in system (min)
Li^+ A	2.80	Start	Sarcosine	78
Li^+ B	3.00	Glutamate	Valine	36
Li^+ C	3.02	Alanine	Leucine	24
Li^+ D	3.45	Cysteine	γ-Aminobutyrate	36
Li^+ E	3.30	Phenylalanine	Arginine	150
LiOH (0.3N)	-	-	-	20
Li^+ A (equilibration)	2.80	-	-	50

mixture until all twenty were present at a concentration of 0.5 micromoles per ml in lithium citrate buffer, pH 2.20; 0.2 N lithium citrate, 1% thiodiglycol, and 0.1% phenol. This became the standard calibration mixture.

This standard was then compared to an Amino Acid Standard, Physiological ANB (Pierce Chemical Co., Rockford, IL). This standard contained a total of thirty-six components, all present at 0.5 µmoles per ml, and included acidic, neutral, and basic amino acids, and related compounds (Fig. 3). Calibration constants were based on the results of ten different runs as shown in Table 4. Norleucine, 0.5 µmoles/ml, was added to each sample as an internal standard. The peak-area ratio calibration curve used for sample analysis is shown (Fig. 4).

Assays for Protein Synthesis

A. Cell-free studies. The incubation conditions were similar to those of Hill, et al (10), using mixtures of amino acids at different concentrations and balances. Typical reaction mixtures contained in a final volume of 250 µl; 20 µmoles Tris-Cl (pH 7.4 at 37°C), 2.5 µmoles PEP, 14 µmoles $MgCl_2$, 0.2 µmoles GTP, 0.25 µmoles ATP, 24 µg pyruvate kinase, appropriate amounts of L-[U-^{14}C] leucine or L-[4,5-^{3}H] leucine, L-[^{12}C] amino acids, and either 0.01 g-equivalents of liver or polysomes and enzyme fractions as specified in the particular experiment that is described. The reaction was terminated by the addition of 250 µl of 10% TCA containing 1 mM L-[^{12}C] leucine. The suspension was cooled on ice for approximately 10 minutes and the insoluble material was sedimented by centrifugation. The supernatant was discarded and the precipitate was resuspended in 5% TCA containing 1 mM L-[^{12}C] leucine. This suspension was heated for 10 minutes at 90°C and recentifuged. This

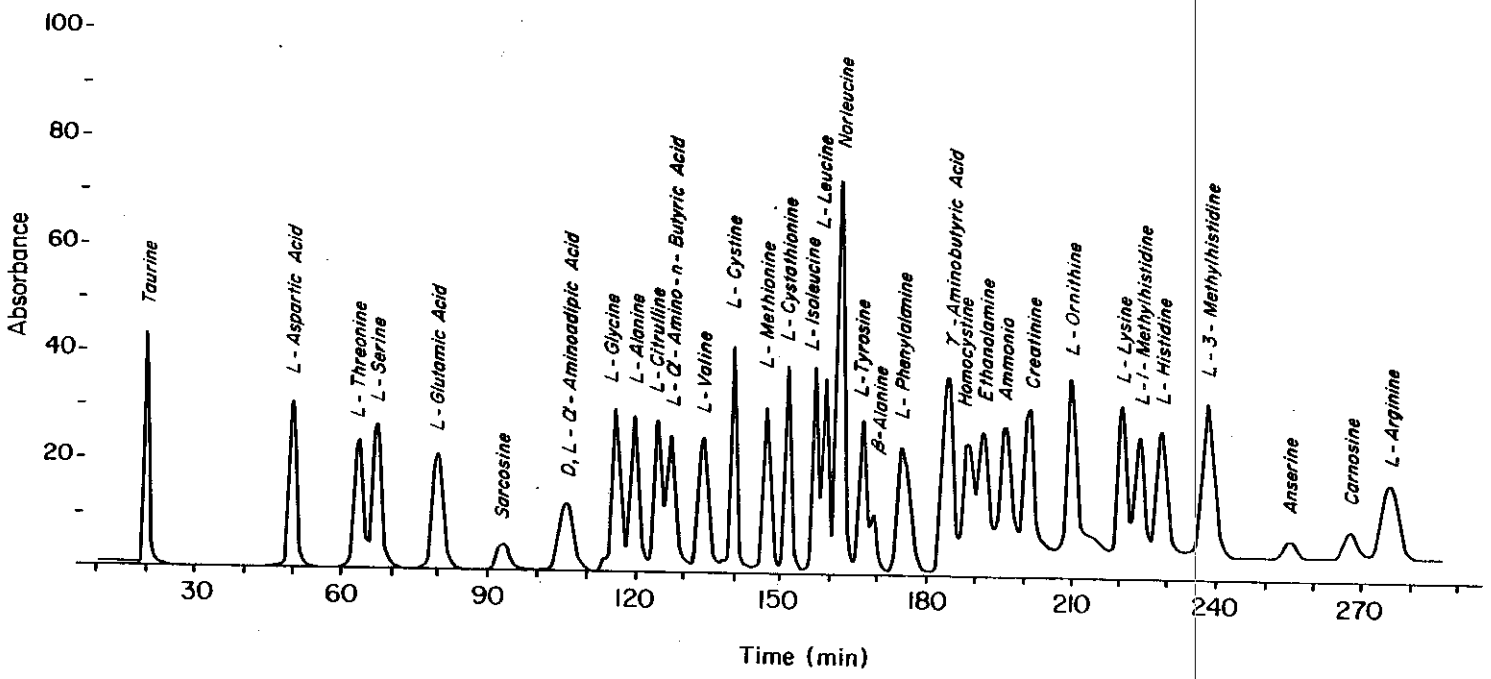

Absorbance
100
80
60
40
20
Taurine
L - Aspartic Acid
L - Threonine
L - Serine
L - Glutamic Acid
Sarcosine
D, L - α - Aminoadipic Acid
L - Glycine
L - Alanine
L - Citrulline
L - α - Amino - n - Butyric Acid
L - Valine
L - Cystine
L - Methionine
L - Cystathionine
L - Isoleucine
L - Leucine
Norleucine
L - Tyrosine
β - Alanine
L - Phenylalanine
γ - Aminobutyric Acid
Homocystine
Ethanolamine
Ammonia
Creatinine
L - Ornithine
L - Lysine
L - τ - Methylhistidine
L - Histidine
L - 3 - Methylhistidine
Anserine
Carnosine
L - Arginine
30
60
90
120
150
180
210
240
270
Time (min)

Table 4

Calibration Constants for Amino Acids Analysis

Amino Acid	t_R[1] $\bar{X}$	t_R[1] SD	k'[2]	$C_{HW_{1/2}}$[3]
ASPARTIC	29.4	4.2	0.54	0.0142
THREONINE	42.6	1.3	1.24	0.0129
SERINE	46.6	1.3	1.45	0.0159
GLUTAMIC	59.2	0.83	2.12	0.0175
GLUTAMINE	64.5	0.68	2.38	0.0125
GLYCINE	94.5	1.65	3.96	0.0153
ALANINE	98.5	1.32	4.17	0.0155
VALINE	112.6	5.60	4.91	0.0149
CYSTEINE	120.6	1.64	5.33	0.0169
METHIONINE	127.6	0.7	5.70	0.0150
ISOLEUCINE	138.2	0.66	6.25	0.0140
LEUCINE	140.5	1.6	6.37	0.0156
NORLEUCINE	143.3	1.3	6.52	-
TYROSINE	148.3	1.6	6.78	0.0145
PHENYLALANINE	156.5	1.58	7.21	0.0153
LYSINE	202.1	1.05	9.61	0.0158
HISTIDINE	210.4	1.58	10.04	0.0156
TRYPTOPHAN	256.4	1.25	12.45	0.0133
ARGININE	258.8	2.7	12.58	0.0147

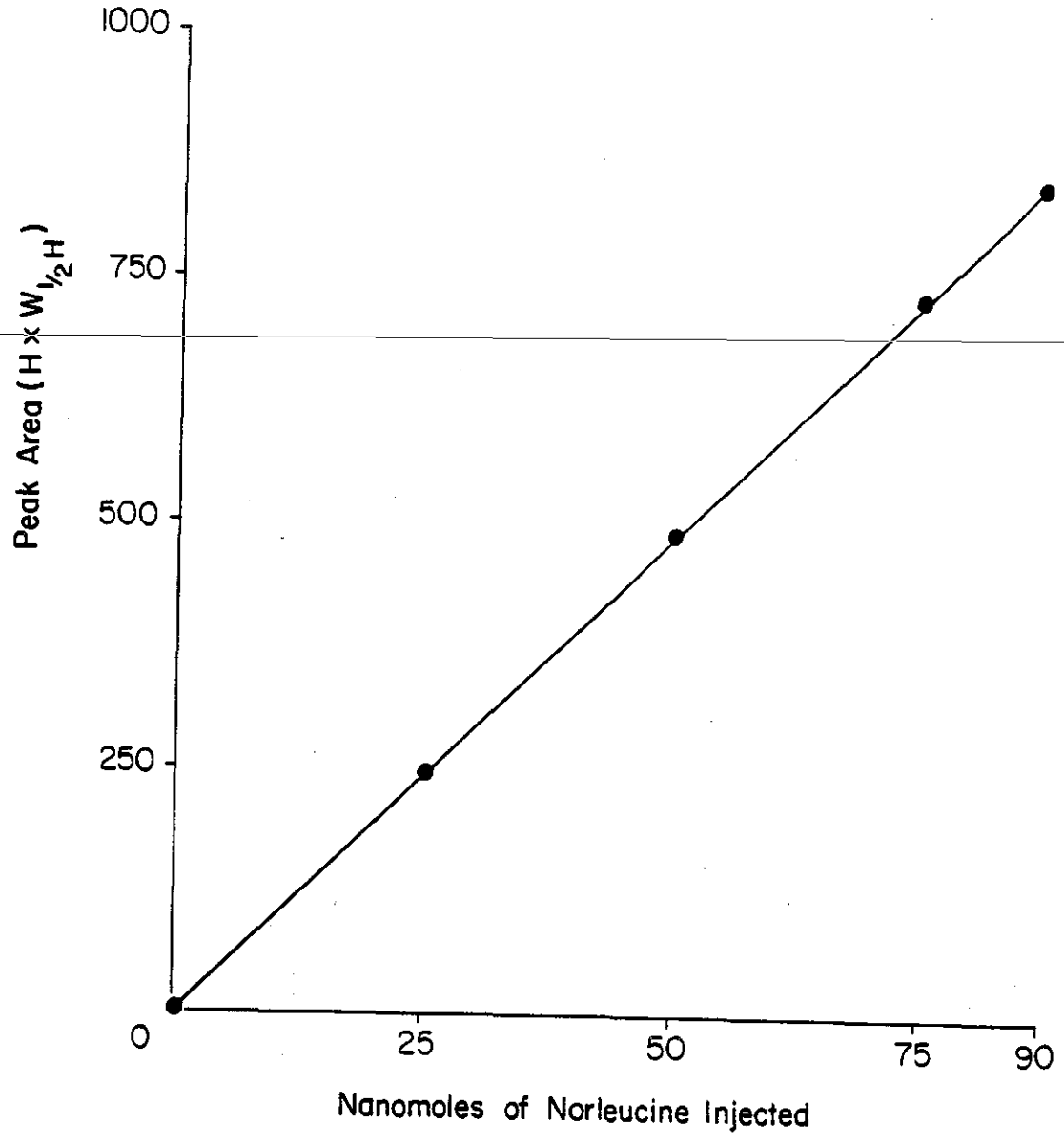
[1] Retention time (t_R) was determined from the elution time (t_o) established as corresponding to the band center of taurine, the first component eluted.

[2] The fundamental liquid chromatography parameter, k, was determined from the equation (49):

$$k' = \frac{t_R - t_o}{t_o}$$

[3] The constant, $C_{HW_{1/2}}$, was determined according to the following equation (49):

$$C_{HW_{1/2}} = \frac{\text{peak height}_{(mm)} \times \text{width at 1/2 height}_{(mm)}}{\text{nanomoles of amino acid injected}}$$


1000
750
500
250
0
Peak Area (H × W1/2 H)
25
50
75
90
Nanomoles of Norleucine Injected

procedure was repeated. Lipid was extracted in a similar manner by treatment of the pellet with ethanol, recentrifugion, a wash with ethyl ether, and a final recentrifugion. This precipitate was dissolved in formic acid. An aliquot of the formic acid protein solution was added to a Beckman BBS-3 solubilizer and a scintillation mixture (Tolmene, PPO and POPOP; 1 liter, 7 gm., 160 mg, Beckman Inst., Palo Alto, CA). The sample was then counted in a CPM-100 scintillation counter (Beckman Instruments, Palo Alto, CA). The counting efficiency for L-[4,5-^{3}H] leucine was 30%, and for L-[U-^{14}C] leucine, 65%.

Essentially, no incorporation of TCA precipitable counts was found at zero time. The system is dependent on the presence of an energy-generating system (Table 5), shows a Mg^{2+} optimum at 9 mM (Fig. 5), demonstrates sensitivity to K^{+} (Table 6), and responds to an inhibitor of protein synthesis (Table 7). Nonspecific binding, as a function of the ratio of L[4,5-^{3}H] leucine to L[U-^{14}C] leucine was also evaluated (Table 8). The extent of amino acid incorporation into protein was determined after an incubation period of 40 min. The incubation time was varied when the rate of protein synthesis was studied. All assay mixtures were prepared in duplicate and incubated at 37^{o}C unless otherwise indicated.

B. Cell culture studies. Mouse fibroblast cells (3T3 4A) were obtained from Dr. R. Holley, La Jolla, CA. Cells were grown in Dulbecco's modification of Eagle's medium (DME) supplemented with 10% heat inactivated, dialyzed calf serum (40,41). Cultures were routinely grown with 1250 U per ml penicillin and 33.3 µg per ml streptomycin sulfate. Cells were plated in plastic tissue culture dishes and grown in a humidified

Table 5

Dependence of Mouse Liver Protein Synthesis on an Energy Generating System

Reaction Conditions	L- [4,5-^{3}H] leucine incorporation	
	CPM[2] Avg ± S_D	% of control
Control (Complete System)[1]	4463 ± 602.00	100
Minus ATP	3006 ± 52.00	69
Minus GTP	3570 ± 250.00	79
Minus ATP, GTP, PEP, PK	68 ± 6.36	2
Minus Balanced Amino Acids	2763 ± 197.00	62

[1] The incubation mixture and reaction conditions are the same as those described in Materials and Methods. Leucine was present at the concentration of 183 µM.

[2] Results are expressed as the average values of duplicate determinations in two separate experiments.

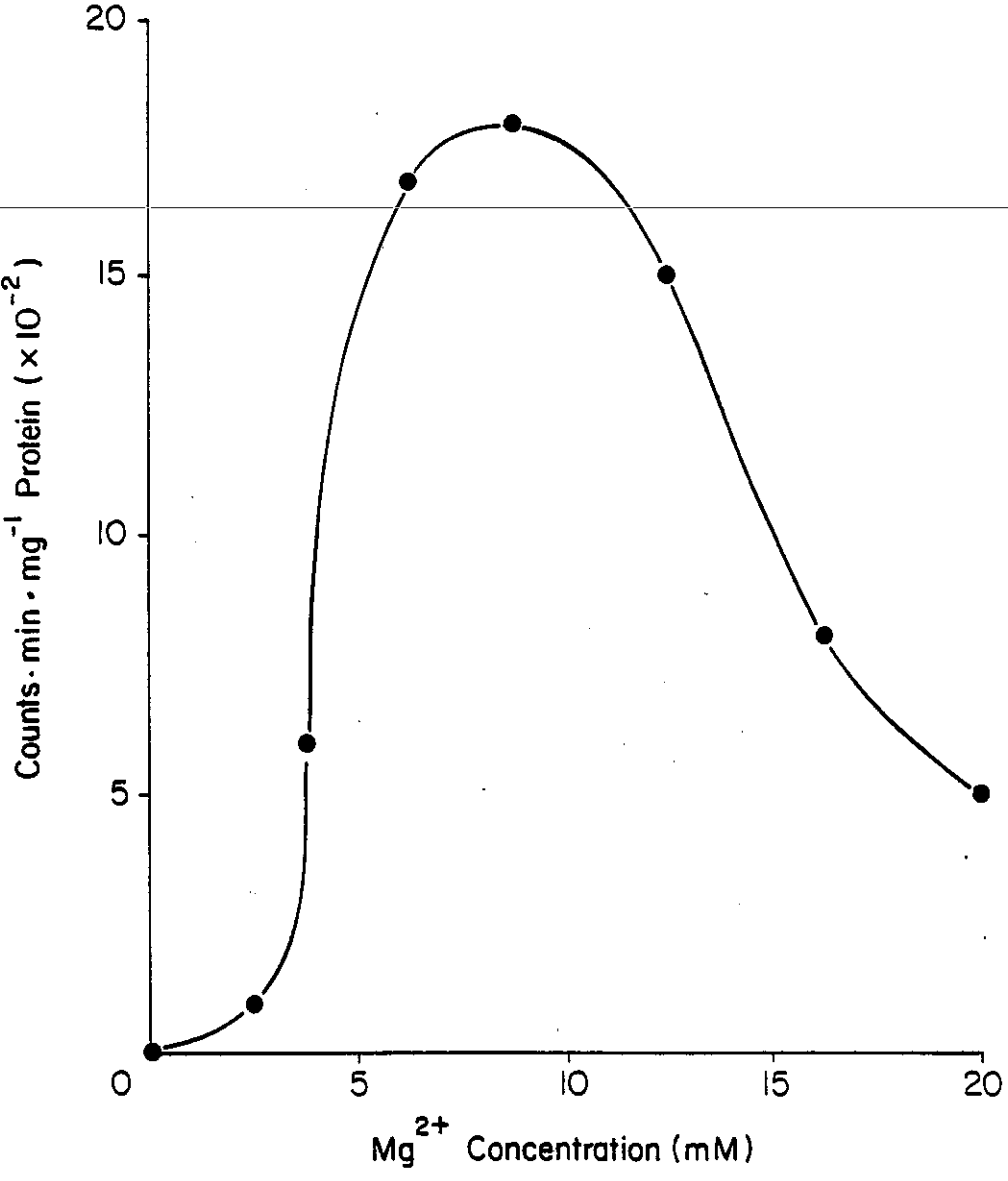

20
15
10
5
0
5
10
15
20
Counts · min · mg^{-1} Protein (×10^{-2})
Mg^{2+} Concentration (mM)

Table 6

Effect of K^+ Concentration on Protein Synthesis in Liver[1]

Experimental Condition[2] K^+ Concentration (mM)	% Leucine Incorporated[3]
7.0	76.75
34.5	85.80
62.5	100.00
117.0	49.82
162.0	42.71

[1] The incubation mix and reaction conditions are the same as those described in Materials and Methods. The 10K-S fraction was used.

[2] Leucine was present at concentration of 183 μM.

[3] Results are expressed as the % of the average value of duplicate determinations in one separate experiment.

Table 7

Edeine Inhibition of Protein Synthesis

Reaction Mixture[1]	L-[4,5-^{3}H] leucine incorporation	
	CPM	%
Complete System	11,503	100
+Edeine[2], 0.5 mM	10,037	87
+Edeine , 1.0 mM	8,725	76
+Edeine , 10.0 mM	612	5

[1] The conditions of this experiment are the same as described in Table 5, except that 2.0 mμmoles of L-[4,5-^{3}H] leucine were added to each assay tube. A 10 K-S fraction was added to the incubation mixture.

[2] Edeine was added to the reaction mixture before incubation at 37°C for 40 min.

[3] Results are expressed as CPM and % of incorporation of L-[4,5-^{3}H] leucine in a single experiment.

Table 8

The Effect of Isotopic Dilution of L-[4,5-^{3}H] leucine on Protein Synthesis in a Mouse Liver System

Leucine Concentration	Specific Activity[2] mCi/μmole	Leucine Incorporated p mole
Leucine - 183 μM	0.518	13.90
	0.259	14.06
	0.106	12.90
	0.052	13.60
Leucine - 5 μM	16.6	4.55
	8.3	4.88
	3.3	4.49
	0.8	4.28

[1] Conditions for the experiment are described in Materials and Methods. The concentration of supernatant (10 K-S) was identical for each condition. The values are average values from a duplicate assay.

[2] The concentration of leucine remained identical for each condition, i.e., 183 μM or 5 μM. However, the ratio of radioactively labelled leucine to cold leucine changed. This represents the specific activity of the L-[4,5-^{3}H] leucine in the incubation medium.

10% CO_2 - 90% air incubator maintained at 37°C. Experimental media consisted of DME medium made equimolar in amino acids (DME-E), and a modified Dulbecco's medium balanced in amino acids (DME-B).

The rate of incorporation of L-[4.5-^{3}H] leucine was determined by adding 20 µCi of leucine (specific activity, 48 Ci/mmole) to each dish at zero time. The media was aspirated from three dishes at various times during the three hours after plating. These were washed twice with Tris-saline, pH 7.4, at 22°C. The dishes were then incubated at 37°C for 30 min in 2 ml of Ca^{2+}- and Mg^{2+}- free trypsin solution. The solution was vigorously pipetted and the dish was scraped with a rubber spatula to ensure removal of the cells. An aliquot of the suspension was then withdrawn and used to determine the number of cells. Another aliquot was transferred to glass tubes and 5 ml of 7.5% TCA was added. The TCA-precipitable protein was retained on Millipore filters (Millipore Corporation, Bedford, Mass), and the radioactivity was determined by counting in a Beckman CPM-100 liquid scintillation counter. Counting efficiency, as determined by the internal standard method, was 38%. Data were expressed as incorporation of leucine per 10^4 cells.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Davis (42) with a water-cooled apparatus manufactured by Buchler Instruments, Fort Lee, NJ).

The gel tubes (12 cm long, 0.8 cm O.D. and 0.6 cm I.D.) were cleaned with detergent solution, rinsed with distilled water and then coated with a solution of 1 part Kodak Photo-Flo per 200 parts of distilled water. The tubes were dried at room temperature and inserted into the

the stopper wells (vertical position).

The composition of the stock solutions was as follows:

(A) 1 N HCl, 48 ml; Tris-base, 36.6 g; N,N,N', N'-tetramethylethylenediamine (TEMED), 0.23 ml and distilled water to complete 100 ml.

(B) 1 N HCl, 48 ml; Tris-base, 5.98 g; TEMED, 0.46 ml and distilled water to complete 100 ml.

(C) Acrylamide, 28 g; N,N'-methylenebisacrylamide (BIS), 0.735 g and distilled water to complete 100 ml.

(D) Acrylamide, 10 g; BIS, 2.5 g and distilled water to complete 100 ml.

(E) Riboflavin, 4 mg and distilled water to complete 100 ml.

(F) Sucrose, 40 g; distilled water to complete 100 ml. Buffer stock: Tris-base, 6.0 g; glycine, 28.8 g and distilled water to complete 1000 ml. Bromophenol Blue: 0.001% solution in distilled water.

The working solutions were as follows:

(A) Small pore solution #1: 1 part of A; 2 parts of C, and 1 part of distilled water.

(B) Small pore solution #2: 0.14 g of ammonium persulfate and distilled water to complete 100 ml. This solution was prepared daily.

(C) Large pore solution: 1 part of B; 2 parts of D; 1 part of E and 4 parts of F.

(D) Buffer solution: The stock buffer was diluted 10 times by adding distilled water.

Stored samples were dialyzed against 200 volumes of stacking (spacer) gel buffer [(0.5 M Tris, 0.48 M Cl^-, pH 6.7; 48 ml 1M HCl, 5.98 g Tris and 0.46 ml TEMED (N,N,N^1,N^1-tetramethylethylenediamine)] made to 100 ml with distilled water prior to electrophoresis. The dialyzed samples

were concentrated to one-fifth the original volume with PVP buffer (polyvinylpyrrolidone in stacking gel buffer) for better gel resolution.

A sample gel solution composed of approximately 0.3 - 0.4 ml of large pore solution was mixed thoroughly in an individual container with an aliquot containing the equivalent of 40,000 cpm of TCA-precipitable material and added to the gel tubes with a capillary pipette. A water layer (about 0.10 ml) was placed on top of the gel and the tubes positioned directly under a fluorescent light or in sunlight until polymerization occurred. Polymerization time varied with the amount of protein used. The tubes were then removed from the light, inverted to eliminate the water layer and rinsed with a few ml of the large pore solution in which the monomer (D) and the sucrose (F) had been replaced by distilled water. The tubes were again inverted and drained.

The spacer gel, composed of the large pore solution, was added to the tubes (about 0.2 ml), layered with water as before, and exposed to light for 20 minutes. Following the polymerization of the spacer, the tubes were inverted (resting on Kimwipes) and rinsed with a mixture of equal volumes of freshly prepared small pore solution 1 and 2. After rinsing, the tubes were filled with an excess of solution so that a "bead" of solution rested on top of each tube (bubbles must be eliminated from the top end). The tubes were protected from strong light until polymerization occurred (approximately 30 min). After polymerization, the parafilm and the stopper wells were removed and the watery solution of the sample end discarded by inverting the gel tubes and touching the open end to absorbent material.

Electrophoresis was started 1 - 2 hrs after the gels were prepared. A drop of the buffer was added to both ends of the gel tubes. The tubes

were then inserted into the grommets of the upper buffer reservoir. A tracking dye (bromophenol blue) was placed in the upper reservoir (0.2 ml of a 0.001% solution) which connected with the cathode of the power supply.

The conditions of electrophoresis using a Buchler power source, (Buchler Instruments, Fort Lee, NJ), were as follows: the sample was run into the spacer gel (1 watt/gel tube) for approximately 15 minutes; power was then increased to 2.0 watts/gel tube for the remainder of the run. A typical electrophoresis run was finished in 2 hrs. The gels were fixed in 7% acetic acid, frozen, sliced into 1 mm sections with a Bio-Rad gel slicer, and individual slices were added to LSC vials. Two ml of 9:1 NCS Tissue Solubilizer (43) were added and the gel slices were incubated at 55^{o}C for 2 hrs in the NCS solubilizer. Fifteen ml of scintillation fluor were added and the radioactivity of the slices was determined. Counting efficiency was determined by quench calculations to be 55% for the ^{14}C channel and 38% for the ^{3}H channel. No counts were recovered in the gels due to non-specific absorption, as shown by control gels in which non-radioactive liver enzyme fractions were coelectrophoresed with ^{3}H and ^{14}C-labelled amino acids. In addition, because of fractionation techniques and specific protein markers, it can be stated that all the counts on the gel were in protein.

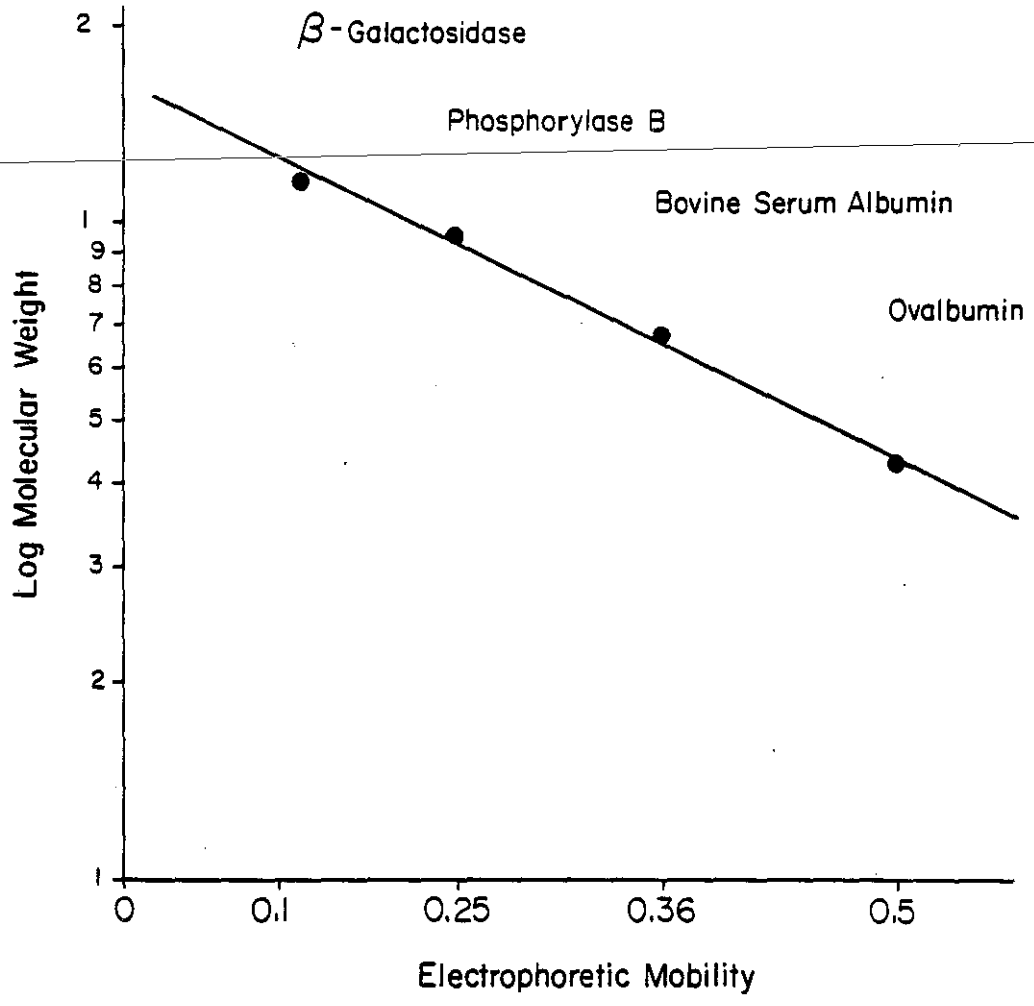
In some experiments it was necessary to run samples containing a large amount of protein. A second method was devised in order to use samples that do not polymerize under normal conditions. After the gel tubes were inserted in the stopper wells, 0.3 ml of large pore solution was added with the aid of a capillary pipette. Water was layered on top of the gel and the tubes were placed directly under light. After

polymerization (15 - 20 min), the tubes were drained as described above. The gel was then layered with 0.6 ml of the large pore solution containing the sample. Water was used again to cover the top and the tubes were exposed to light for 1 hr. At the end of this period, 0.3 ml of large pore solution was layered on top of the gels and the samples again exposed to light until polymerization of the upper gel occurred. The rest of the procedure was the same as described above.

Albumin synthesis was verified by separation of fractions on sodium dodecyl sulfate (SDS) Biophore precast gels (Bio-Rad Lab., Richmond, CA) and by its isolation using immunoprecipitative techniques (Bio-Rad, Bulletin 1038). Gel and sample conditions were varied according to the types of proteins being studied. The specific conditions are described for each experiment discussed. High Molecular Weight Protein Standards (40,000 - 250,000 Daltons) and Low Molecular Weight Protein Standards (10,000 - 100,000 Daltons) obtained from Bio Rad Laboratories, Richmond, CA (Fig. 6), were used in gel calibration for molecular weight determination and protein identification. Electrophoretic conditions are described in the legend to specific figures and tables.

Gel Staining

Gels, prepared according to the method of Ornstein and Davis (42,44) for nondissociating conditions, were fixed and stained by treatment of the gel with a solution of 0.05% Coomassie Brilliant Blue R-250 in 7% aqueous acetic acid. Gels were stained for 1 hr. After staining, they were destained with a 7% acetic acid solution in Hoeffer destaining apparatus (Hoeffer Equip., San Francisco, CA) until a clear background was obtained.


β-Galactosidase
Phosphorylase B
Bovine Serum Albumin
Ovalbumin
Log Molecular Weight
2
1
9
8
7
6
5
4
3
2
1
0
0.1
0.25
0.36
0.5
Electrophoretic Mobility

SDS gels were fixed with a solution of 40% isopropanol and 10% acetic acid. They were then stained using a solution of 0.05% Coomassie Brilliant Blue R-250 in 10% acetic acid and 10% isopropanol. The destaining procedure was as described above except that 10% acetic acid and 10% isopropanol solution were used.

Localization of Albumin and Globulin Fractions in the Gel

Albumin and globulin fractions were coelectrophoresed on one gel from each experiment (gels were always run in triplicate). Gels were stained, scanned with a Transidyne General Densitometer (Model TG 2970, Transidyne General Corp., Ann Arbor, Mich), destained and sliced. The appearance of both albumin and globulin fractions allowed for the determination of REMAG values (relative electrophoretic mobility of albumin and globulin, Sayre, unpublished). This was done for each experiment to normalize the gels from one experiment to another.

Immunoprecipitation of Albumin

The precipitations were performed in 1.0 ml plastic microfuge tubes. In a typical precipitation of albumin, 100 µl of rabbit anti-mouse albumin antisera (Miles Laboratories, Elkardt, Indiana) and 20 µl of 10% sodium deoxycholate in distilled water were added to the tubes. The sample (50 - 100 µl), in phosphate buffer (10 mM sodium phosphate - 15 mM NaCl, pH 7.5) and buffer were then added to make a total volume of 300 µl. The contents of the tube were mixed and incubated at 37^{o}C for 60 min. The tubes were then centrifuged at 7,000 x g (Fisher, Model 59) for 4 min. The supernatant was aspirated and the precipitate resuspended in 300 µl of buffer. The suspension was then centrifuged at 7,000 x g and the

resulting supernatant discarded. This washing procedure was repeated. The washed precipitate was dissolved in 100 µl of formic acid and quantitatively transferred to a 20 ml liquid scintillation vial. Finally, 2 ml of solubilizer (Beckman, BBS-3) and 10 ml of toluene-based scintillation fluid were added, the contents thoroughly mixed, and the vial placed in the liquid scintillation counter (Beckman, CPM-100) for counting. All reported results are based on duplicate assays. Data were evaluated as follows:

Controls, containing 50 µl of mouse serum albumin (Miles Laboratories, Elkhart, Ind) and an equivalent amount of antibody plus labelled free amino acid, were included in provide a control for nonspecific trapping of radio-label. In addition, an equivalent amount of unlabelled incubation mixture containing liver homogenate and labelled free amino acid were mixed with antibody as a similar control for non-specific absorption. Cross reactivity and immunochemical identity were evaluated by rocket and crossed immunoelectrophoresis procedures as described in subsequent sections. The optimum amount of antiserum necessary for complete precipitation of albumin by either single (Fig. 7) or double immunoprecipitation (Table 9) was determined.

Double immunoprecipitation experiments became the routine assay for quantitation of albumin. A second antibody, goat anti-rabbit IgG (Miles Laboratories, Elkhart, Ind), was added at specified concentrations after the incubation step and then reincubated for the same period of time as described above. It made no difference whether the samples were incubated 1 hr or overnight at 37^{o}C.

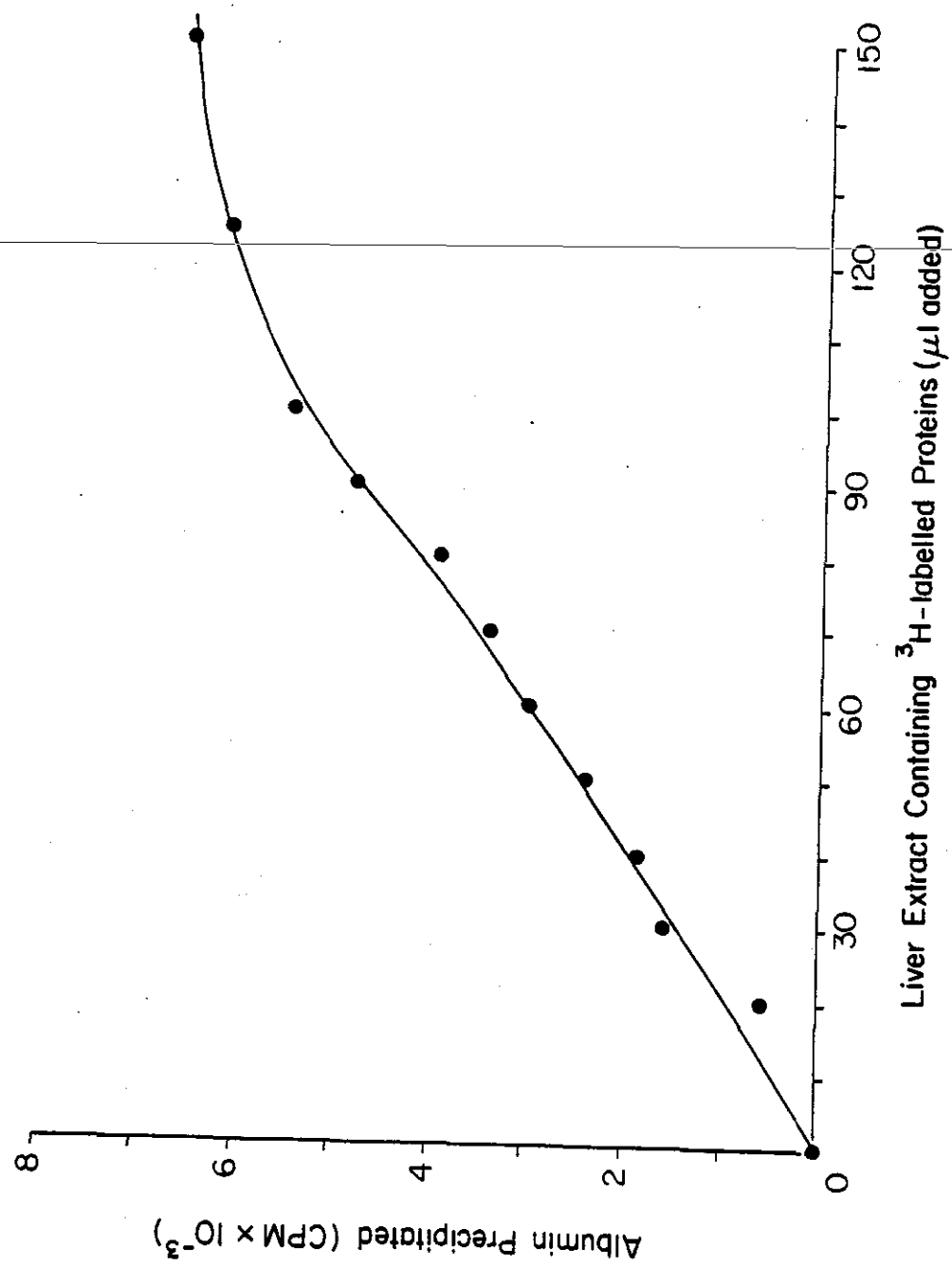

Albumin Precipitated (CPM × 10⁻³)
0
2
4
6
8
30
60
90
120
150
Liver Extract Containing ³H-labelled Proteins (μl added)

Table 9

Dependence of Immunoprecipitation on Goat-Antirabbit IgG Concentration[1]

Ratio of Antigen:Antibody	Immunoprecipitate CPM	%
10	1623	15
5	1887	18
2	3273	31
1	5282	49
0.5	7463	70
0.25	10683	100
0.16	9715	91
0.10	6184	58

[1] The reaction conditions are the same as described in Materials and Methods except that 50 µl of rabbit anti-mouse albumin antisera was added to each tube rather than 100 µl. The results are based on a single experiment.

Immunoelectrophoresis

The immunoelectrophoretic conditions were a modification of those described by Weeke (45), and are based upon the electrophoretic migration of antigens in antibody-containing gel. Individual precipitates are formed for each antigen-antibody system present. The area enclosed by these precipitates is proportional to the antigen/antibody ratio. The method is therefore suitable for immunological identification as well as quantitation and cross reactivity determinations. The procedure described is the one used for rocket immunoelectrophoresis (46).

The 100 x 100 mm glass plates were cleaned with a non-ionic detergent, rinsed with distilled water and dried in a dust-free atmosphere.

The stock buffer solutions and agarose were prepared as follows:

A. Buffer 1: 20.6 g sodium diethylbarbiturate, 4.0 g diethylbarbituric acid, 1.0 g sodium azide dissolved in hot distilled water. The solution was allowed to cool, and the pH was adjusted to 8.6 with 0.5 N NaOH. The final volume was 1000 ml. This was diluted 1 part buffer to 4 parts water for a working solution.

B. Agarose: 1 g agarose powder was added to 100 ml of barbital buffer. The solution was boiled gently with frequent stirring. The prepared agarose was stored at $4^{o}C$ in 15 ml screw-capped tubes until further use.

The plates were coated with a thin layer of agarose and dried under a current of warm air. This became necessary to insure adhesion of the gel to the plate. It was also necessary if the gel was to be stained. The agarose was heated to $55^{o}C$. An amount of antibody, as indicated in the specific experiment, was added to the heated agarose and mixed gently. Air bubbles must be avoided at this step. A layer of agarose

gel was then cast on the surface of the plate. The 100 x 100 mm gel was approximately 1.5 mm thick.

The surface of the gel was cooled to 10-14^{o}C prior to and during the sample run; tap water was passed through copper coils emersed in an ice bath.

Sample wells were formed in the gel before sample application by means of a gel puncher attached to a vacuum source. The wells were spaced 10 mm apart for the rocket technique of Laurell (46), and 10 - 20 mm from the edge of the plate. The plate was placed on the surface of the electrophoresis cell, gel side up, and contact with the buffer was made by the use of gel wicks. The sample, in a volume of less than 10 µl was applied into the gel well, and the run immediately begun at a voltage of 2.0 volts/cm. The run continued overnight.

The current was switched off and the plate was removed from the electrophoresis cell. The sample wells were filled with water and the plate was covered with a wet filter paper and 8 - 10 pieces of dry filter paper. A pressure greater than 1 kg was applied to the glass plate for 30 min. This was one of the most critical steps necessary in obtaining a good gel plate. Following the pressure application, the gel plate was immersed in 0.1M NaCl (10 min) and rinsed twice in distilled water. These washing steps were then repeated. The gel was dried under a current of warm air until the gel became transparent.

The plate was then placed in staining solution (Coomassie Brilliant Blue R-250, 5 g; 95% ethanol, 450 ml; acetic acid, 100 ml; distilled water, 450 ml) for 3 minutes. The plate was then destained in an ethanol-acetic acid water solution (250 ml:100 ml:450 ml). The plates were rinsed briefly with water and dried.

Crossed immunoelectrophoresis is basically similar to the rocket technique except that it is a two dimensional procedure. The first step in crossed immunoelectrophoresis, i.e., agarose gel electrophoresis, is performed in the conventional way. After electrophoresis in the first direction, two parallel cuts 5 mm apart are made in the gel. The cuts are in the same direction as the first dimensional run. The gel between the cuts was removed and transferred to another gel plate; agarose gel containing antibody was added to the remainder of the new plate and electrophoresis was run in the second dimension, perpendicular to the gel strip for the first dimensional run. This is not a quantitative procedure, but rather, a highly sensitive method for the evaluation of immunological microheterogeneity (47).

Determination of Protein Concentration

Protein concentration were determined by the method of Lowry, et al. (48).

Analysis of Data

Incorporation Experiments

The incorporation of amino acids into proteins was determined by measuring the amount of radio-labelled amino acid incorporated into total acid precipitable protein. The results of these experiments are reported as pmoles of total leucine incorporated. Counting efficiency was determined by the external-standard ratio method as well as by the internal standards method. A program for processing data was written for a TI 59 calculator (Texas Inst., Houston, TX) (Appendix A). The calculation of the total amount of leucine incorporated was accomplished by entering the count rate, as cpm, the dilution factor and the external

standard ratio. Duplicate results were processed automatically and the results were reported as the mean and standard error of the mean.

Cell culture studies

The incorporation of amino acids into proteins by cells in culture was determined as previously described for the cell-free system studies. The number of cells in culture was determined by means of a Coulter Counter.

Analysis of data from polyacrylamide gel electrophoresis

A program has been devised for the computer to process and plot the radioactive data obtained from single or double labelling experiments (Appendix B). The program was written in Fortran IV for use with the Burroughs 6700 Computer. The line-printer was used as a plotting device as well as for presentation of computed data.

Calibration data for efficiency calculation, quench correction and energy crossover for counting ^{3}H and ^{14}C samples were obtained by the internal standard method and the external standard ratios method. The same liquid scintillator was used for sample analysis as was used to obtain calibration data.

Evaluation of data from amino acid analysis

A program was written for the quantitative evaluation of the chromatograms obtained from the automatic amino acid analyzer. The program described is written for a Texas Instruments TI 59 calculator. The concentration of amino acid can be calculated in ng·100 ml^{-1} plasma and in mM. The program is divided into two sections: one to calculate the calibration constants from a standard, and the other to determine the

concentration of the amino acids in the sample. An internal standard, norleucine, was added to each sample at the same concentration as was present in the standard. The ratio of each amino acid to the internal standard normalized all values with respect to sample data and to values obtained from standard chromatograms.

Reference compounds were selected on the basis of their isolation from other components and their peak symmetry. Each reference amino acid was eluted by a different buffer. The reference compounds were the following: aspartic acid, glycine, valine, norleucine, and lysine.

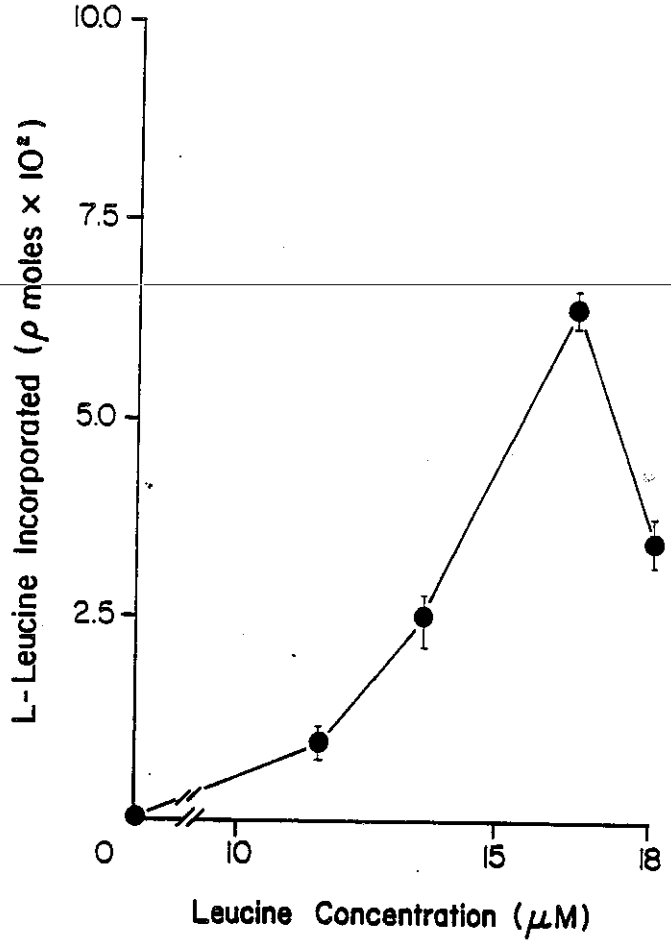
Peak area measurements were made manually; the peak height and the width at one-half the peak height were obtained using an Alltech Peakometer (Alltech Inc., Arlington Heights, IL). This data was then processed automatically by the TI 59 program.

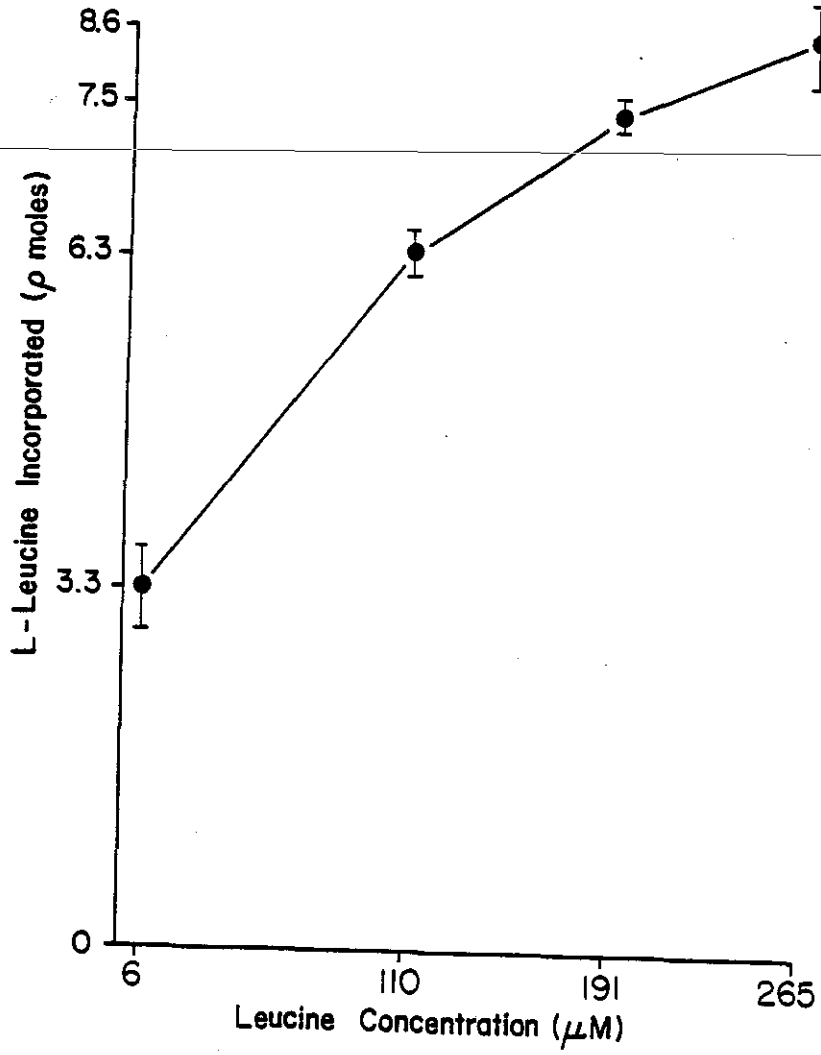
RESULTS

Effect of Leucine Concentration on Amino Acid Incorporation by Liver Tissues

The dependence of protein synthesis upon leucine concentration is shown in Fig. 8. These results were observed with a highly fractionated system in which microsomes were separated from the supernatant fraction (40 K-S, 40 K-M). Amino acid incorporation was optimal at a leucine concentration of 16.5 μM. Higher concentrations of leucine inhibited synthesis.

A more intact system, in which a 10,000 xg supernatant fluid was used (10 K-S), showed no inhibition of amino acid incorporation by added leucine over a concentration range from 0 to 265 μM (Fig. 9). The physiological mean and concentration range of leucine (183 ± 23 μM) were based on plasma values.


10.0
7.5
5.0
2.5
0
10
15
18
L-Leucine Incorporated (ρ moles x 10²)
Leucine Concentration (μM)


8.6
7.5
6.3
3.3
0
L-Leucine Incorporated (ρ moles)
6
110
191
265
Leucine Concentration (μM)

Effect of Balanced and Equimolar Amino Acid Mixtures on Amino Acid Incorporation

The incorporation of amino acids into proteins by a cell-free system with an equimolar mixture of amino acids or a balanced mixture of amino acids is shown in Fig. 10. Incorporation was significantly greater with balanced mixtures than with equimolar mixtures of amino acids over the range of concentrations which included the physiological range. Inhibition of protein synthesis did not occur with balanced mixtures as total amino acid concentrations were increased. Inhibition was observed with equimolar amino acid mixtures at increased total concentrations. This effect was observed over a range of total amino acid concentration from less than 1 millimole per liter to greater than 8 millimoles per liter. The same finding was observed at each concentration tested. Although the total amounts of leucine incorporated into protein increased as the concentration of amino acids increased, the major effect upon the amount of leucine incorporated was the balance of amino acids.

The effects of balanced and equimolar amino acid mixtures were then compared to those with no amino acids added to the incubation mixtures. The incorporation of L-[4,5-^{3}H] leucine into acid insoluble counts was significantly greater with the balanced amino acid mixture than with the equimolar amino acid mixture (Table 10).

Incorporation of L-leucine into Protein of 3T3 Cultured Cells

The incorporation of L-[4,5-^{3}H] leucine into TCA-precipitable protein by 3T3 cells grown in amino acid test media is shown in Fig. 11. Murine fibroblast cells were grown in Dulbecco's modified Eagle's medium (DME), Dulbecco's modified Eagle's medium equimolar in amino acids (DME-E), and

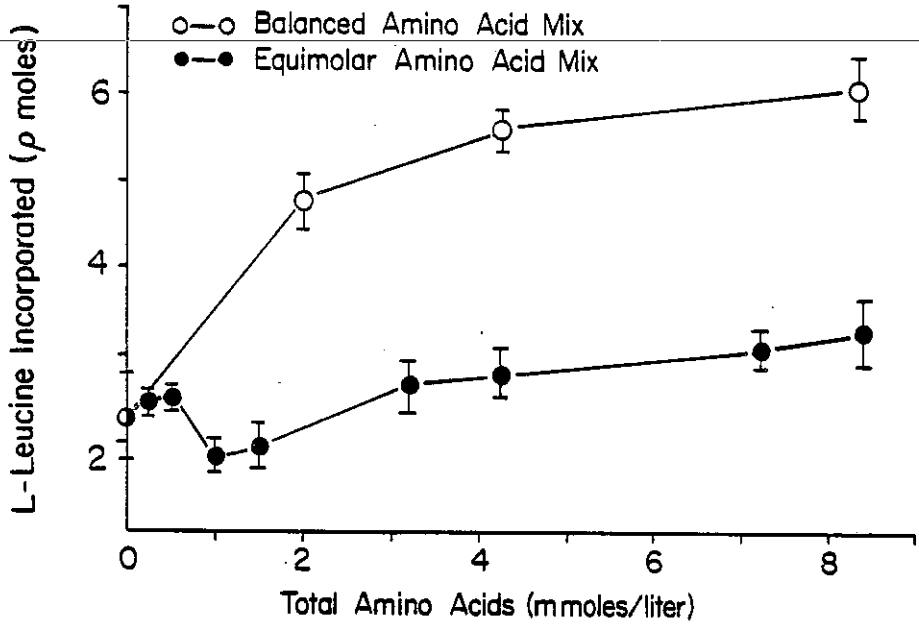

o—o Balanced Amino Acid Mix
●—● Equimolar Amino Acid Mix
L-Leucine Incorporated (ρ moles)
2
4
6
0
2
4
6
8
Total Amino Acids (mmoles/liter)

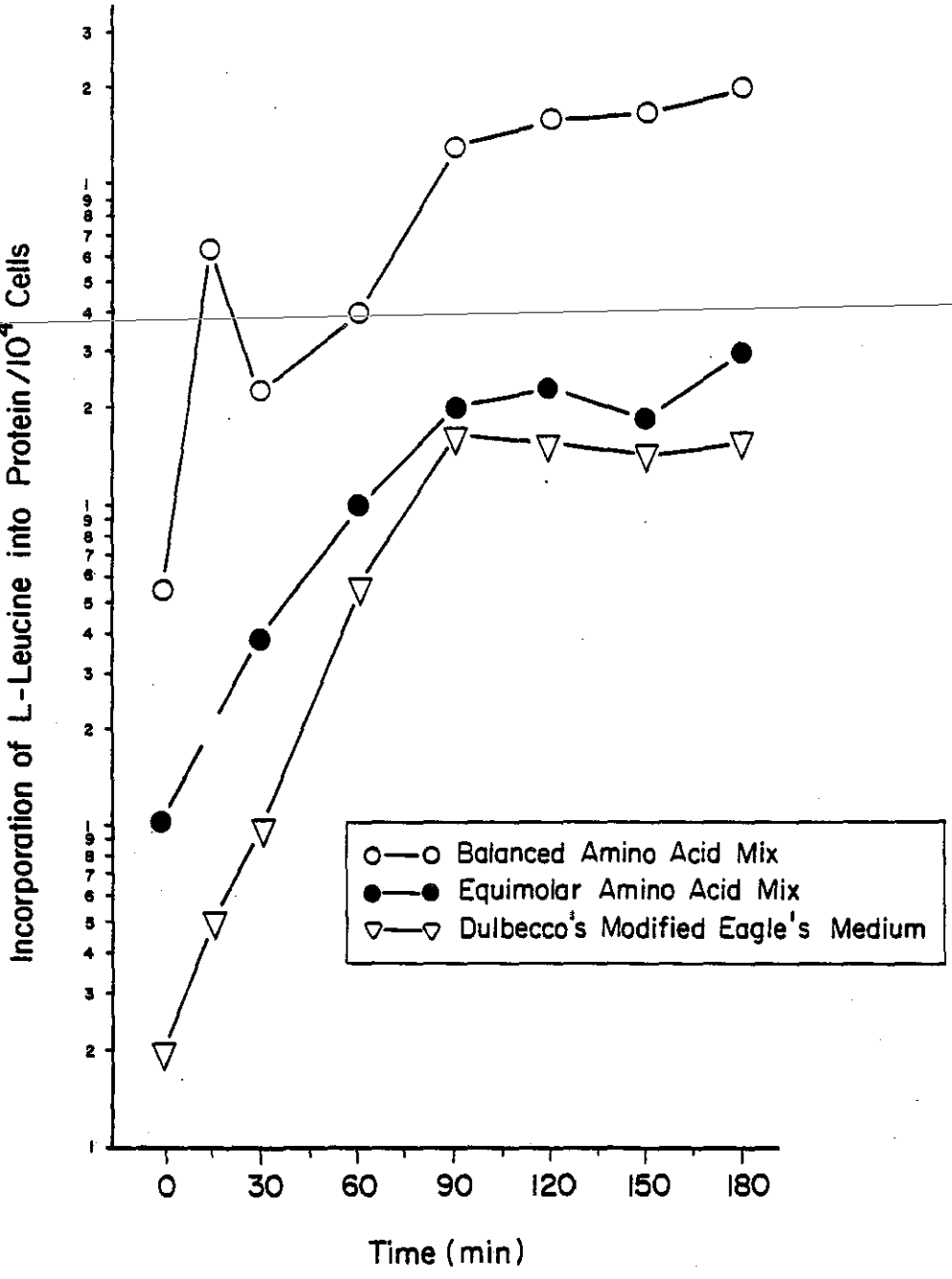
Table 10

Effects of a Balanced Amino Acid Mixture and of an Equimolar Amino Acid Mixture on the In Vitro Incorporation of L-[4,5-H^3] Leucine into TCA-Precipitable Protein[1]

	Radioactivity Incorporated			
Experimental Condition	CPM/Incubation Duplicate Assays[2]	Zero Time	Net CPM	% Change
	Control (Avg)			
No Amino Acids Added	25,562 20,398	2,201	20,779	0
Equimolar Mixture of Amino Acids	20,838 23,396	2,268	19,848	-4.48
Balanced Mixture of Amino Acids	35,616 36,932	2,134	34,140	+64.30

[1] Incubation conditions were as described in Materials and Methods, except that the total incorporating system contained 340 µg of liver 10 K-S fraction. Leucine was limiting at 5 µM.

[2] Results are presented as the average of duplicate experiments, each with duplicate determinations.


Incorporation of L-Leucine into Protein /10⁴ Cells
Time (min)
0
30
60
90
120
150
180
Balanced Amino Acid Mix
Equimolar Amino Acid Mix
Dulbecco's Modified Eagle's Medium

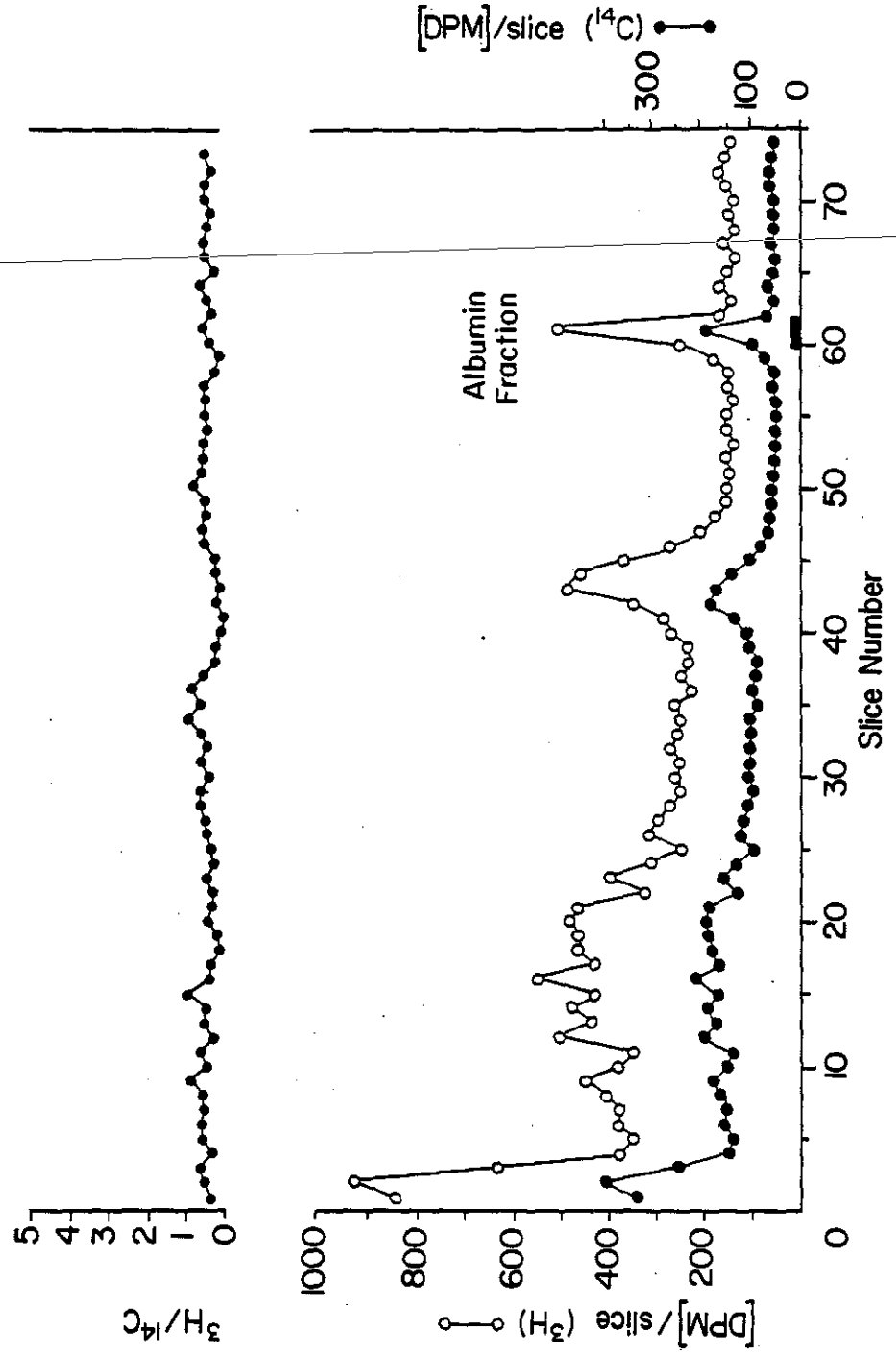
Dulbecco's modified Eagle's medium balanced in amino acids (DME-B). The balanced amino acid mixture gave significantly greater incorporation of leucine into protein than did either DME or the DME-E. These results agree with those obtained with the cell-free systems (Fig. 10, Table 10).

Analysis of Protein Synthesis by Polyacrylamide Gel Electrophoresis

The effect of amino acid balanced on the patterns of protein synthesized *in vitro* and *in vivo* is shown in Figs. 12A and 12B. The $^{3}H/^{14}C$ ratio, which indicates the differential synthesis of protein, showed that albumin synthesis was influenced more by the addition of a balanced rather than an equimolar mixture of amino acids (Fig. 12B). The *in vivo* pattern of proteins synthesized was used as a reference profile. The correlation between proteins synthesized *in vitro* and *in vivo* was closer with balanced mixtures than it was with equimolar mixtures of amino acids. Differences were also observed in gel slices 42 - 60 and in gel slice 10. The relative proportion of counts found in the albumin fraction is shown in Table 11 for the three different conditions.

The Effect of Tryptophan Concentration upon Total Protein Synthesis

Tryptophan was found to have a different effect depending upon whether an equimolar or a balanced mixture of amino acids was used. Increases in tryptophan concentration had very little effect upon the amount of tritiated leucine incorporated into total precipitable protein over a concentration range from 1 to 124 micromolar when an equimolar mixture of amino acids was used (Fig. 13). When a physiologically balanced mixture of amino acids was used, however, the amount of protein synthesized increased with increasing concentrations of tryptophan over a range of 1 to 25 micromolar. Further increases in tryptophan


[DPM]/slice (^{14}C)
300
100
0
Albumin Fraction
70
60
50
40
30
20
10
0
Slice Number
1000
800
600
400
200
0
[DPM]/slice (^{3}H)
5
4
3
2
1
0
^{3}H/^{14}C

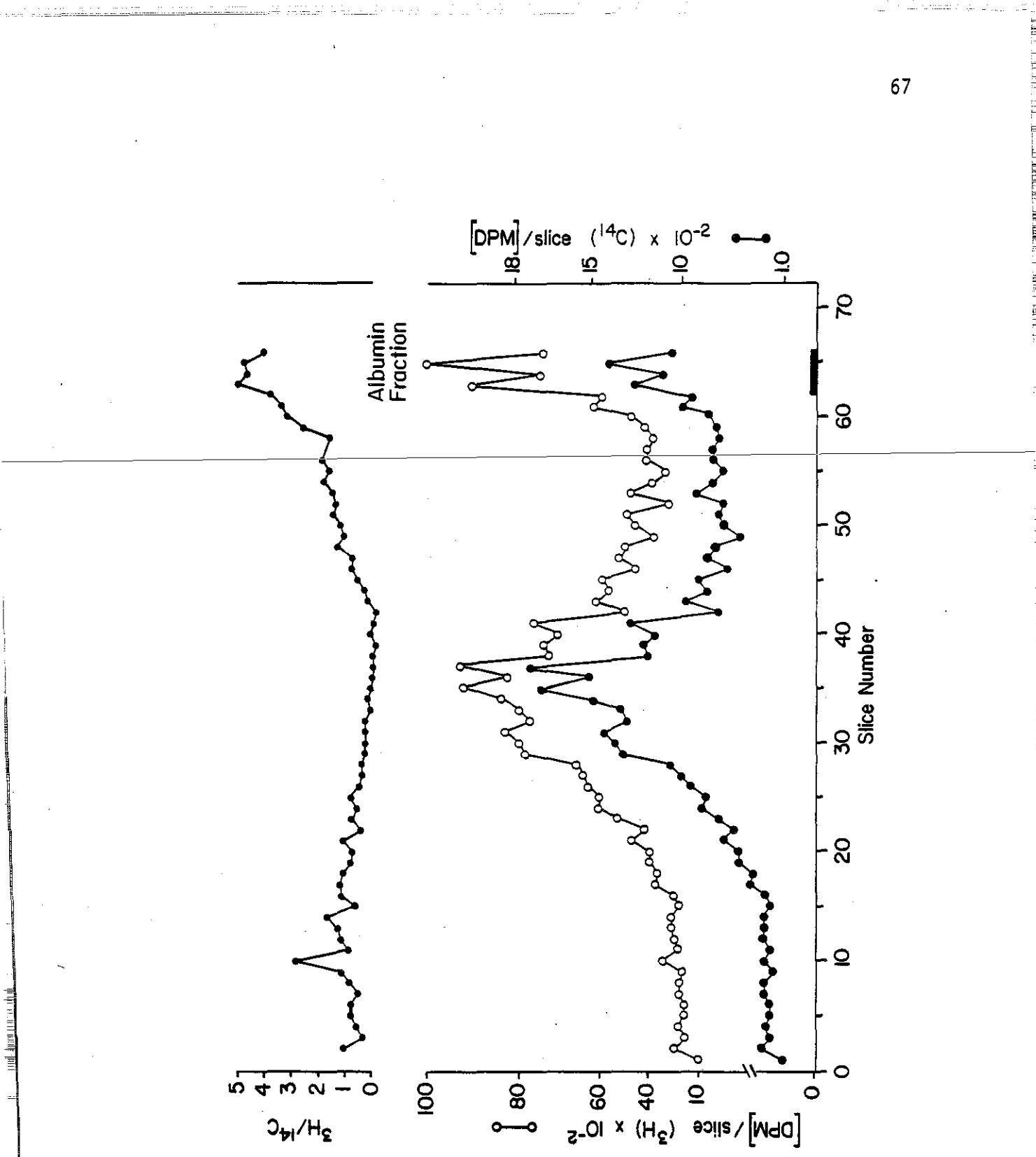
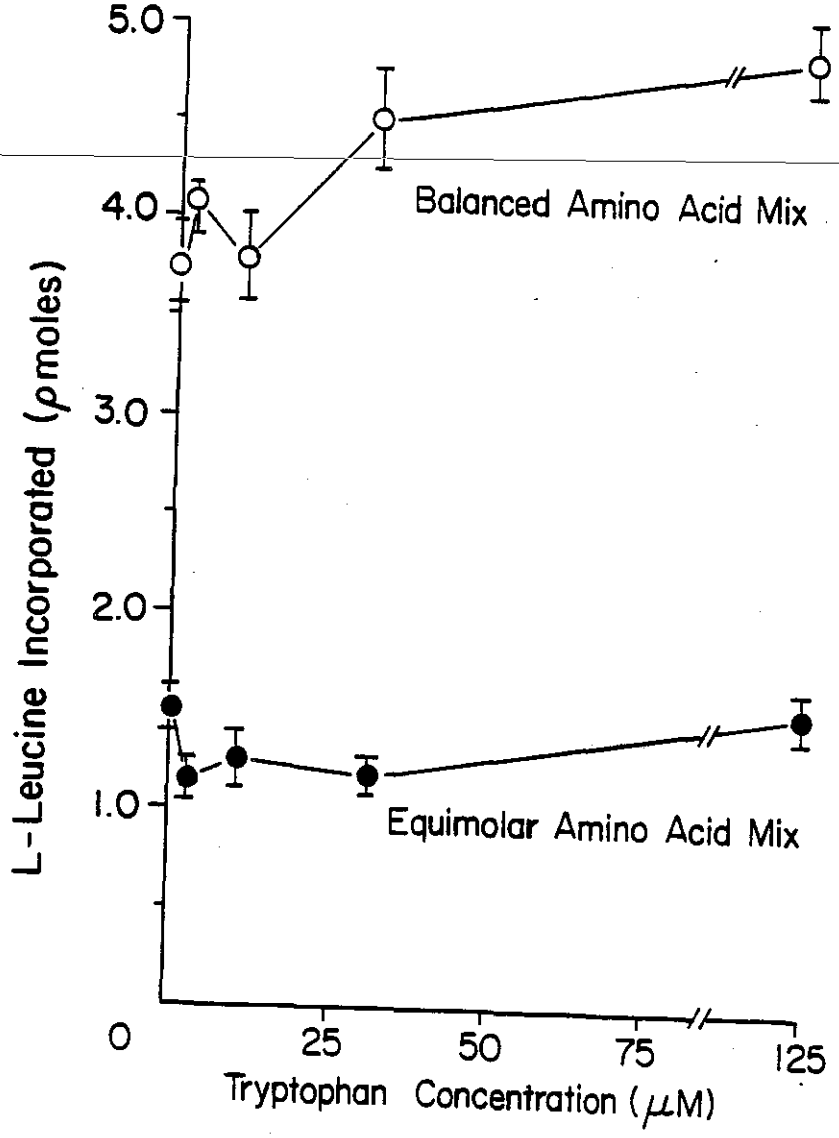

[DPM]/slice (^{14}C) x 10^{-2}
Albumin Fraction
[DPM]/slice (^{3}H) x 10^{-2}
^{3}H/^{14}C
Slice Number

Table 11

Relative Proportion of Counts
in Albumin Fraction

Experiment Description	% of Radioactivity in Albumin Fraction
In Vivo	30.1 ± 1.5
In Vitro - Balanced	16.3 ± 1.1
In Vitro - Equimolar	11.1 ± 1.2

The percent of counts in the major albumin fraction was determined by computer analysis of the areas beneath each peak using Simpson's Rule. Each experiment was the result of duplicate runs. The values are expressed as the mean ± standard deviation.


5.0
4.0
3.0
2.0
1.0
0
L-Leucine Incorporated (ρmoles)
Balanced Amino Acid Mix
Equimolar Amino Acid Mix
25
50
75
125
Tryptophan Concentration (μM)

concentration up to 218 micromolar showed no increases in the amount of protein synthesized.

The amount of tritiated leucine incorporated into total precipitable protein was approximately four times as great with a balanced mixture of amino acids as it was with an equimolar mixture. Despite these differences, changes in tryptophan concentration were without major effect upon the amount of protein synthesized when the concentrations were varied within the physiological range for this amino acid. The normal plasma value of tryptophan was estimated to be 62 micromolar (36).

Tryptophan was found to be without effect upon the incorporation of radio-labeled amino acids over the physiological concentration range when all other amino acids, including leucine, were present in a physiologically balanced mixture (Table 12).

Effects of Tryptophan and Leucine Concentration on the Synthesis of the Albumin Fraction

The effects of tryptophan concentration on the synthesis of the albumin fraction was highly dependent on the experimental conditions. A balanced amino acid mixture increased synthesis of the albumin fraction by 24% (Table 13) when tryptophan was 62 μM, and leucine was limiting at 5 μM. However, at higher concentrations of tryptophan, synthesis of the albumin fraction was depressed. In the presence of an equimolar amino acid mixture, the higher tryptophan concentration stimulated the synthesis of albumin but 60 μM tryptophan had no effect. Albumin synthesis was not affected by changing tryptophan levels when the concentration of leucine was 183 μM, as shown in Table 14. This indicates that leucine concentration, rather than amino acid balance or tryptophan concentration, is a more important determinant of protein synthesis in liver tissue.

Table 12

The Effect of Tryptophan Concentration on Protein Synthesis in the Presence of 183 μM Leucine

Experimental Conditon	Tryptophan Concentration (μM)	P Moles Leucine Incorporated
Equimolar Mixture of Amino Acids	2	12.7
	60	12.6
	124	12.8
Balanced Mixture of Amino Acids	2	14.2
	60	12.4
	124	12.8

The conditions of this experiment are as stated in Figure 10 except that 340 μg of 10 K-S were added in place of microsomes and enzyme fraction. The values reported are averages based upon duplicate assays for each point.

Table 13

Effect of Tryptophan Concentration on the Synthesis of the Immunoprecipitable Albumin Fraction with Leucine Limiting

Experimental Condition Tryptophan Concentration (μM)	P Moles Leucine Incorporated	
	Balanced Amino Acids	Equimolar Amino Acids
2	0.131 ± 0.004[1]	0.105 ± 0.004
60	0.142 ± 0.005	0.108 ± 0.003
120	0.124 ± 0.002[2]	0.119 ± 0.003[3]

[1] Significantly different from control at 60 μM tryptophan, $p < .05$.

[2] Significantly different from control at 60 μM tryptophan, $p < 0.01$.

[3] These values are expressed as the mean ± standard deviation. (n = 4)

Table 14

Effect of Tryptophan Concentration on the Synthesis of the Immunoprecipitable Albumin Fraction with Leucine Physiological[1]

Experimental Condition Tryptophan Concentration (μM)	pMoles Leucine Incorporated	
	Balanced Amino Acids	Equimolar Amino Acids
2	3.59 ± 0.304	3.34 ± 0.077
60	3.03 ± 0.247	3.76 ± 0.346
120	3.73 ± 0.848	3.77 ± 0.134

[1] The values reported are based on the mean ± standard deviation. (n = 4)

This is supported by experiments in which albumin fraction synthesis was studied as a function of leucine concentration (Table 15). If the concentration of leucine was physiological rather than limiting, the quantity of albumin fraction synthesized was 20 times greater regardless of amino acid balance. Albumin fraction synthesis, in comparison to total synthesis of protein, also changed as a function of leucine concentration. When leucine was physiological, albumin accounted for 6.0% of protein synthesized. Albumin synthesis accounted for only 2.5% of total protein synthesis when leucine was limiting, and a balanced amino acid mixture was added; if an equimolar mixture of amino acids was added, albumin synthesis accounted for 1.8% of the total protein synthesized. These results were obtained by the direct immunoprecipitation method.

The Effect of Primary and Secondary Immunoprecipitation on Interpretation of Levels of Albumin Synthesis

The methods used for quantitation of albumin were evaluated because of a disparity among the values reported in the literature. The results of direct and indirect immunoprecipitation are shown in Table 16. The precipitation curve is presented in Fig. 14. Less than 12% of the radioactive albumin was precipitated by the use of rabbit anti-mouse albumin serum. Indirect immunoprecipitation of the tissue extract increased the amount of albumin precipitated by 8-fold to 30 percent of the total. The ratio of albumin synthesis to total protein synthesis in the liver is not precisely known and values from 0.56% to 33% have been reported in the literature (50). Hill, et al. (10) have reported a value of 31% for the albumin fraction using a similar 10,000 x g supernatant system.

Table 15

Albumin Synthesis as a Function of Leucine Concentration and Balance of Amino Acids[1]

Experimental Condition	Leucine Concentration			
	Limiting (1 μM)		Physiological (183 μM)	
	% of Total Protein Synthesized	p Mole Leucine Incorporated	% of Total Protein Synthesized	p Mole Leucine Incorporated
Equimolar M Mixture of Amino Acids	1.8	0.107 ± 0.0029	5.8	3.30 ± 0.247
Balance Mixture of Amino Acids	2.5	0.141 ± 0.0048[2]	5.8	3.76 ± 0.346

[1] Results are presented as picomoles of leucine incorporated into the immunoreactive albumin fraction. Albumin was precipitated by rabbit anti-mouse albumin IgG (performed as described in Materials and Methods). Values are presented as mean ± standard deviation. (n = 4)

[2] Significantly different, $p < 0.01$.

Table 16

Comparison of Primary and Secondary Immunoprecipitation Methods for the Measurement of Albumin

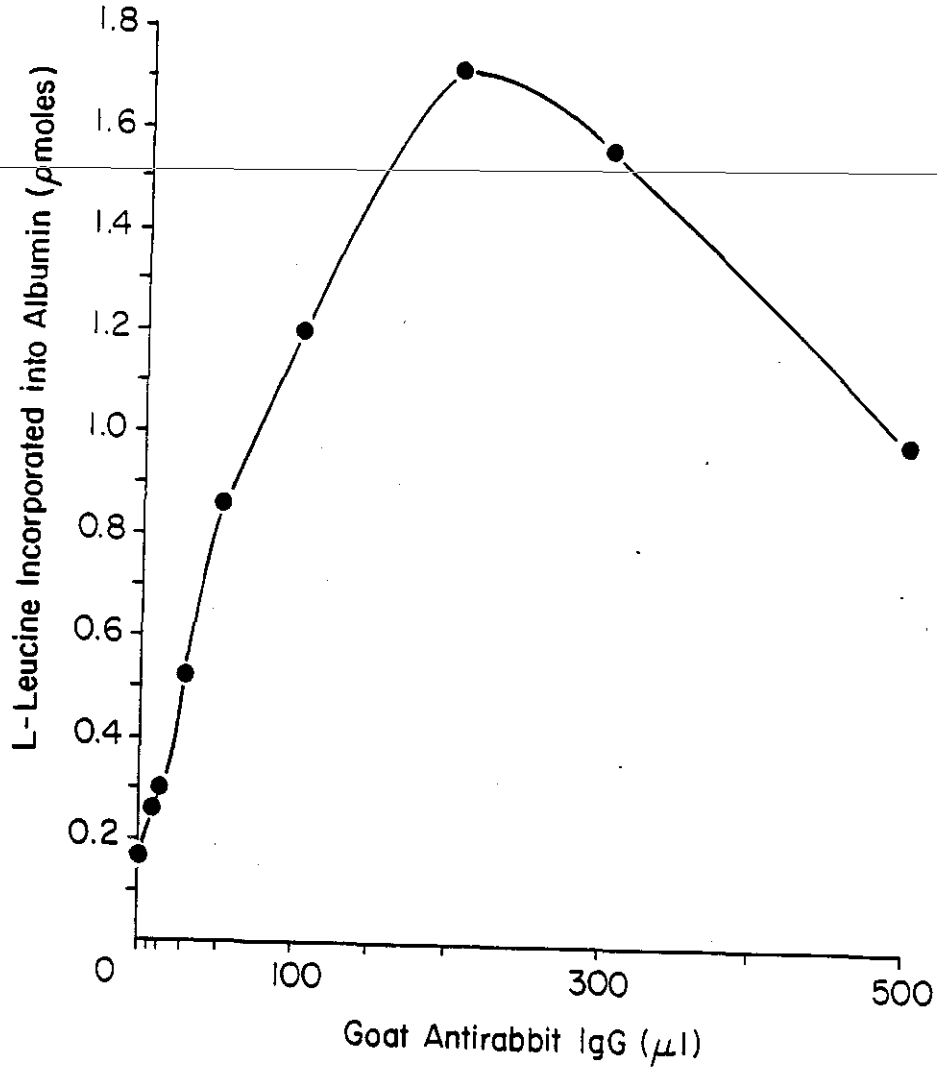
Experimental Conditions	pMoles Leucine Incorporated into Albumin	% Total Protein Synthesis[3]
Direct Immunoprecipitation by Rabbit Antimouse Albumin Serum[1]	0.160 ± 0.008[4]	2.7
Indirect Immunoprecipitation of Rabbit Antimouse Albumin Complex by Goat Antirabbit IgG[2]	1.72 ± 0.05	30.22

[1] The precipitates were performed in 1.5 ml plastic microfuge tubes. In the direct immunoprecipitation reaction, 100 µl of incubation mix for protein synthesis was withdrawn at 40 min. and added to the microfuge tube containing 100 µl of rabbit anti-mouse albumin serum, 20 µl of 10% sodium deoxycholate in distilled water and 80 µl of phosphate buffer (10 mM sodium phosphate - 15 mM NaCl, pH 7.5). The total volume was 300 µl. Controls were included to account for non-specific binding. The tubes were incubated for 1 hr at 37°C and processed as described in Materials and Methods.

[2] The indirect immunoprecipitation method was similar to the direct method except that after 1 hr. incubation at 37°C, 200 µl of goat anti-rabbit antisera was added to each tube, plus 800 µl of phosphate buffer. The tubes were mixed and reincubated for 30 min. at 37°C. Processing was done as described in Materials and Methods.

[3] Leucine was limiting at 5 µM in protein synthesis assay. Total protein synthesis was 5.69 ± 0.16 pmole leucine incorporated into protein.

[4] Results are presented as the mean ± standard deviation. (n = 3)

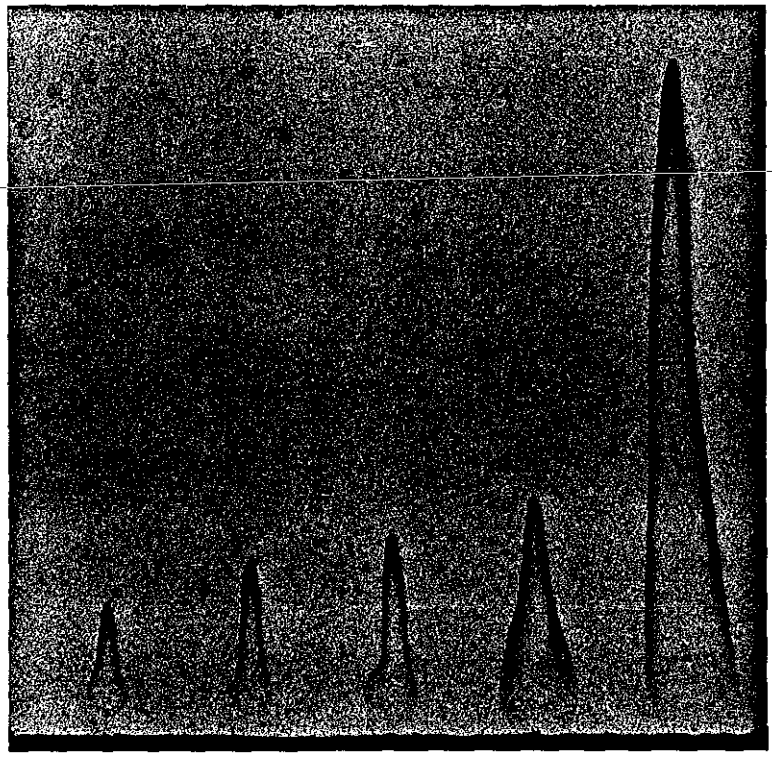

L-Leucine Incorporated into Albumin (μmoles)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
100
300
500
Goat Antirabbit IgG (μl)

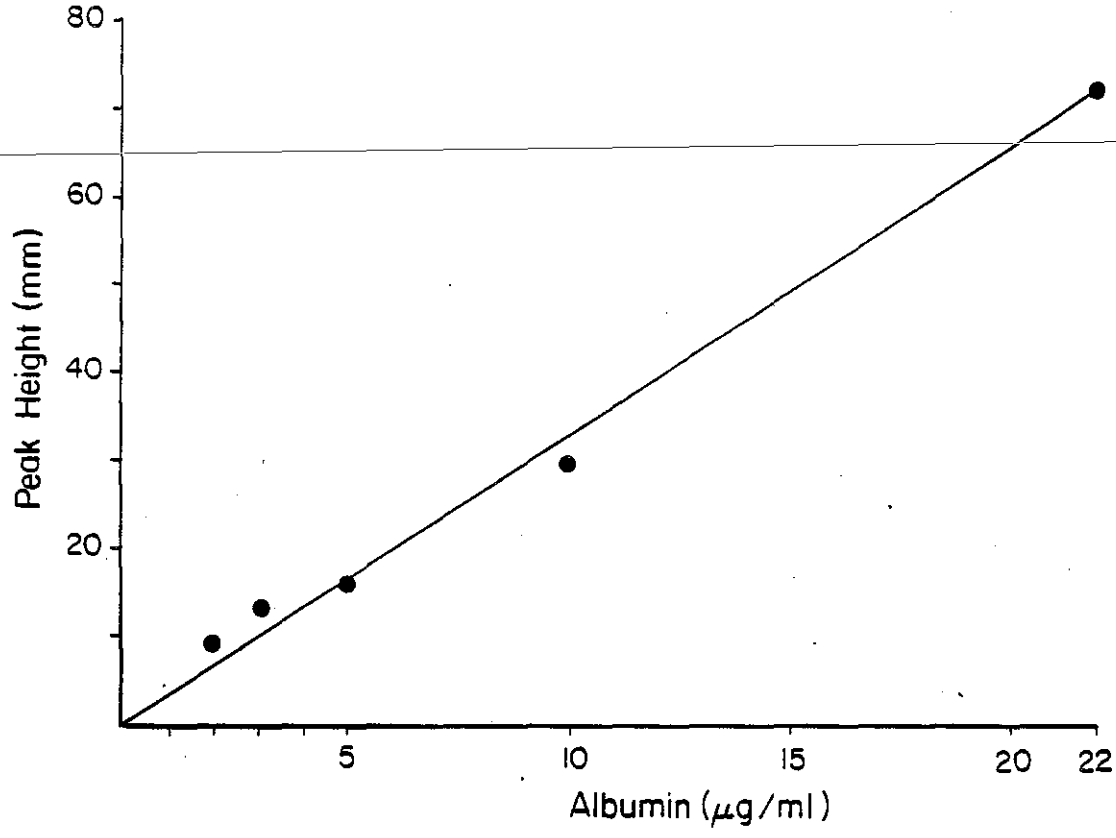
This immunoprecipitated albumin fraction was further characterized by extensively washing the precipitate in buffered saline and analyzing the fraction obtained by crossed and rocket immunoelectrophoresis as well as dissociating gel electrophoresis. Rocket immunoelectrophoresis showed that the albumin from the liver extract reacted quite specifically to albumin antiserum as shown in Fig. 15. No additional precipitin lines were found within the rockets at various concentrations of antigen. The precipitation heights were linear with respect to the amount of albumin (Figure 16). This suggests that the antisera was monospecific for albumin.

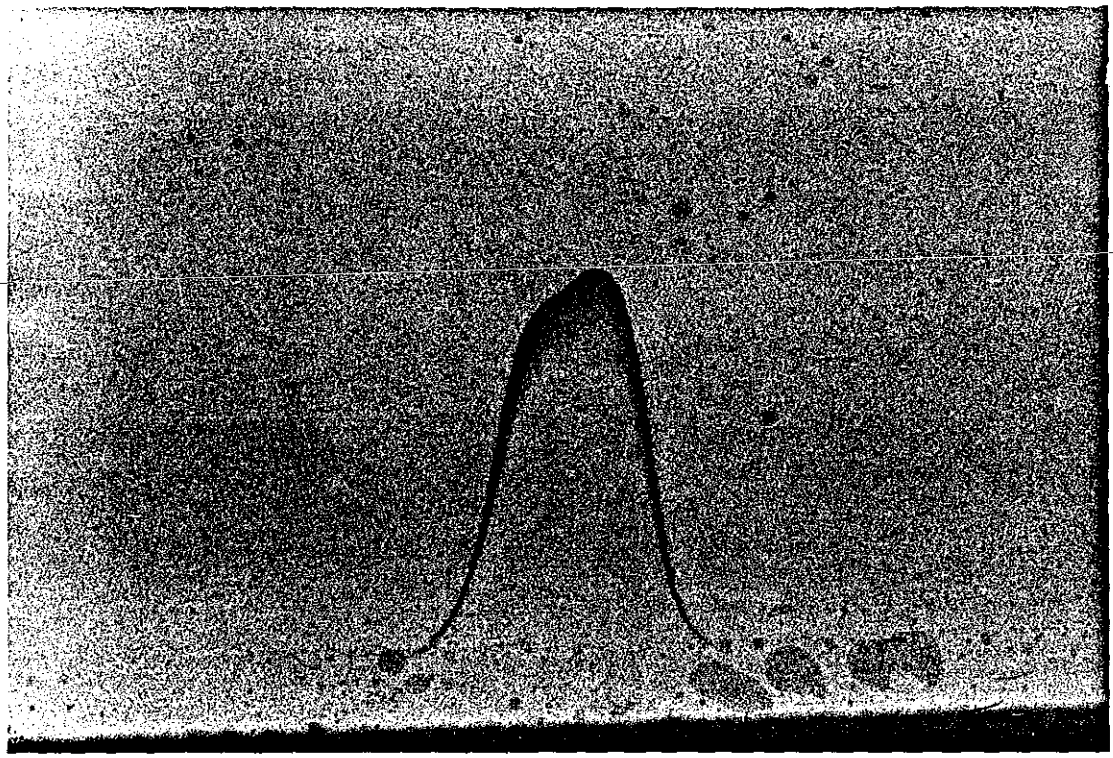
Crossed immunoelectrophoresis was used as a more sensitive immunoelectrophoretic technique to evaluate antigen-antibody specificity. The standard mouse albumin fraction contained more than one component that reacted with monospecific albumin (Figure 17).

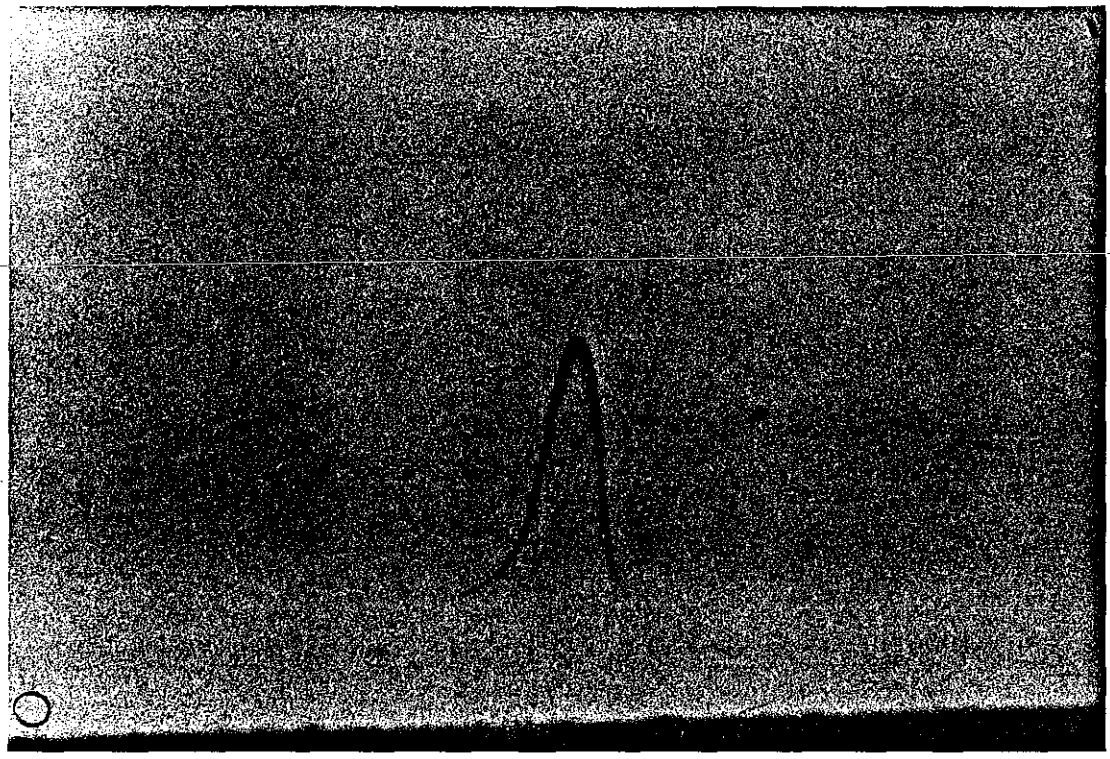
The liver extract showed a single precipitation peak that was not symmetrical, indicating a reaction of partial identity with a minor component present in the liver extract (Figure 18). The signs of partial identity with this method are: a) partial fusion of precipitates; b) identical precipitate morphology; and c) similar electrophoretic ability under non-dissociating conditions.

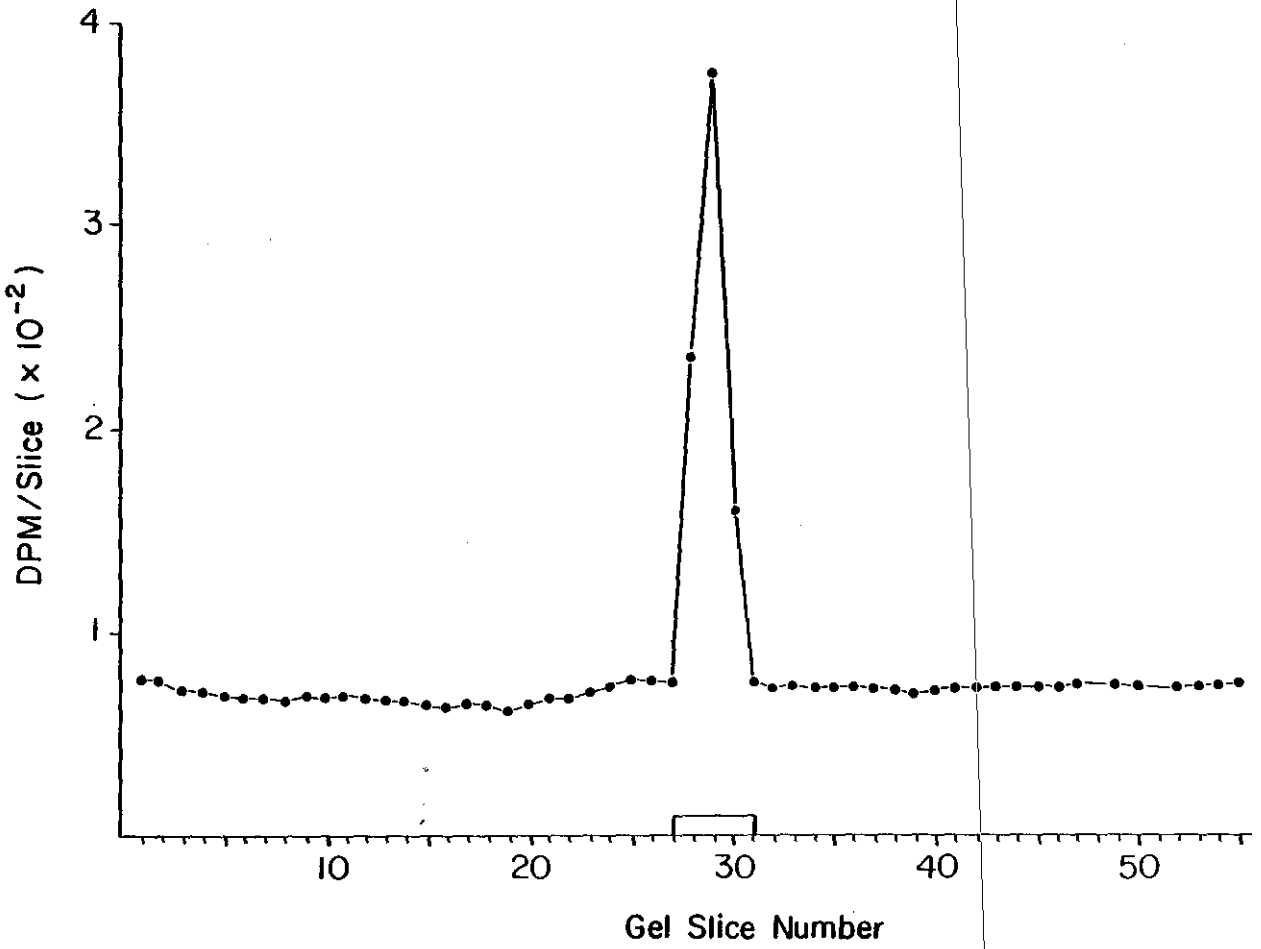
The radiolabelled albumin fraction obtained from the 10 K-S by the direct immunoprecipitation method was electrophoresed under conditions dependent upon the molecular weight of the protein. The gel profile for the fraction is shown in Figure 19. Identification of the radioactive component as albumin was based on comparison to the relative mobility of a mouse albumin standard immunoprecipitated with the monospecific antisera. The radiolabelled albumin fraction obtained from the 10 K-S by




80
60
40
20
Peak Height (mm)
5
10
15
20
22
Albumin (μg/ml)





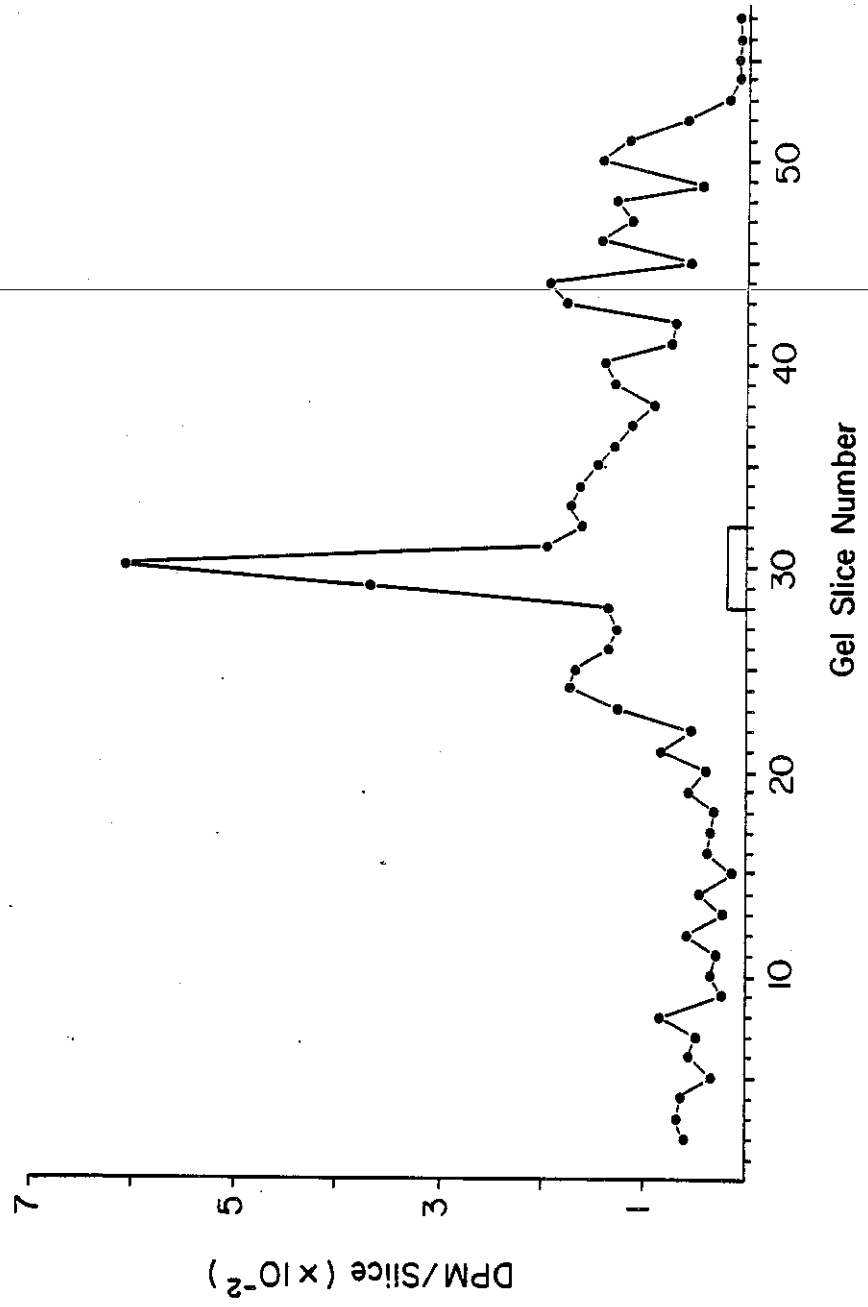

4
3
2
1
DPM/Slice (x 10^{-2})
10
20
30
40
50
Gel Slice Number

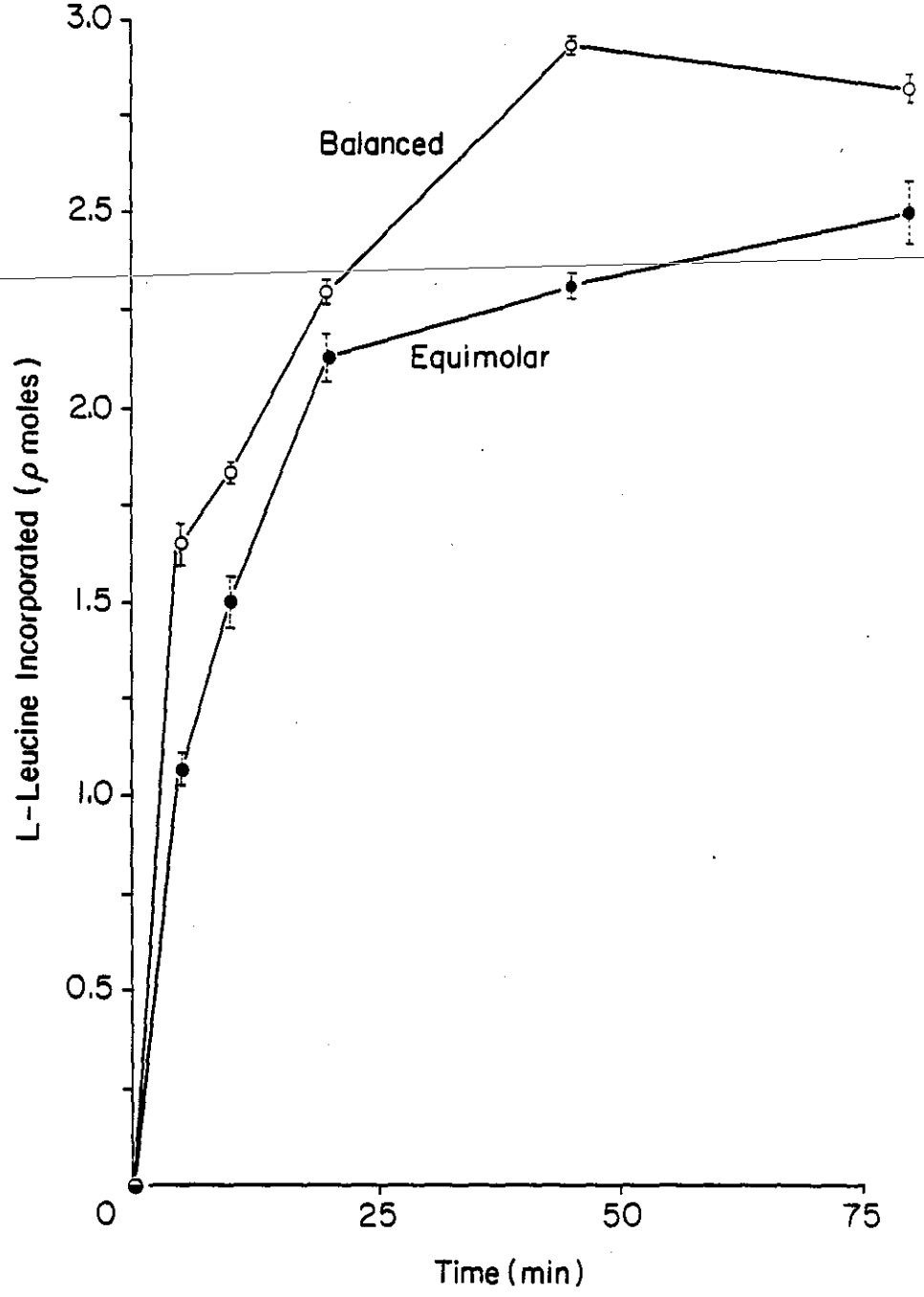
the indirect immunoprecipitation method was electrophoresed under identical conditions (Fig. 20). Several minor peaks with increased mobility (gel slices 38-55) were observed in addition to the major albumin peak. There was also a minor component (gel slices 23-37) with decreased mobility in the immunoprecipitable fraction. The fractions with increased mobility are low molecular weight fractions (less than 68,000 daltons) and most likely represent nascent peptide chains with immunoreactive sites. It is believed that these fractions are partially synthesized albumin peptide chains, because of their antigenic similarity to albumin. The fractions with slightly decreased mobility have an average molecular weight of rat liver pre-proalbumin (51-53), and the faster migrating species, i.e., proalbumin (approximately 70,000 daltons). The direct and indirect immunoprecipitation methods were both internally consistent, as shown in Table 16.

These results indicate that quantitation of albumin by immunoprecipitative techniques is not valid. Rather, reference to the immunoprecipitated material as the albumin fraction is acceptable. This provides an indirect assessment of the quantity of albumin present in various forms, i.e., either as albumin, pro-albumin, or pre-proalbumin, and nascent peptide chains.

The Effect of Leucine Concentration and Amino Acid Balance on the Rate of Protein Synthesis

The rate of protein synthesis was studied as related to the relative concentration of amino acids and the concentration of leucine. When leucine was limiting, the difference in the extent of incorporation was significantly greater at 5 min. and at 40 min. than at any other time (Fig. 21). There was a time dependent change in the rate and extent


DPM/Slice (x 10^-2)
7
5
3
1
10
20
30
40
50
Gel Slice Number

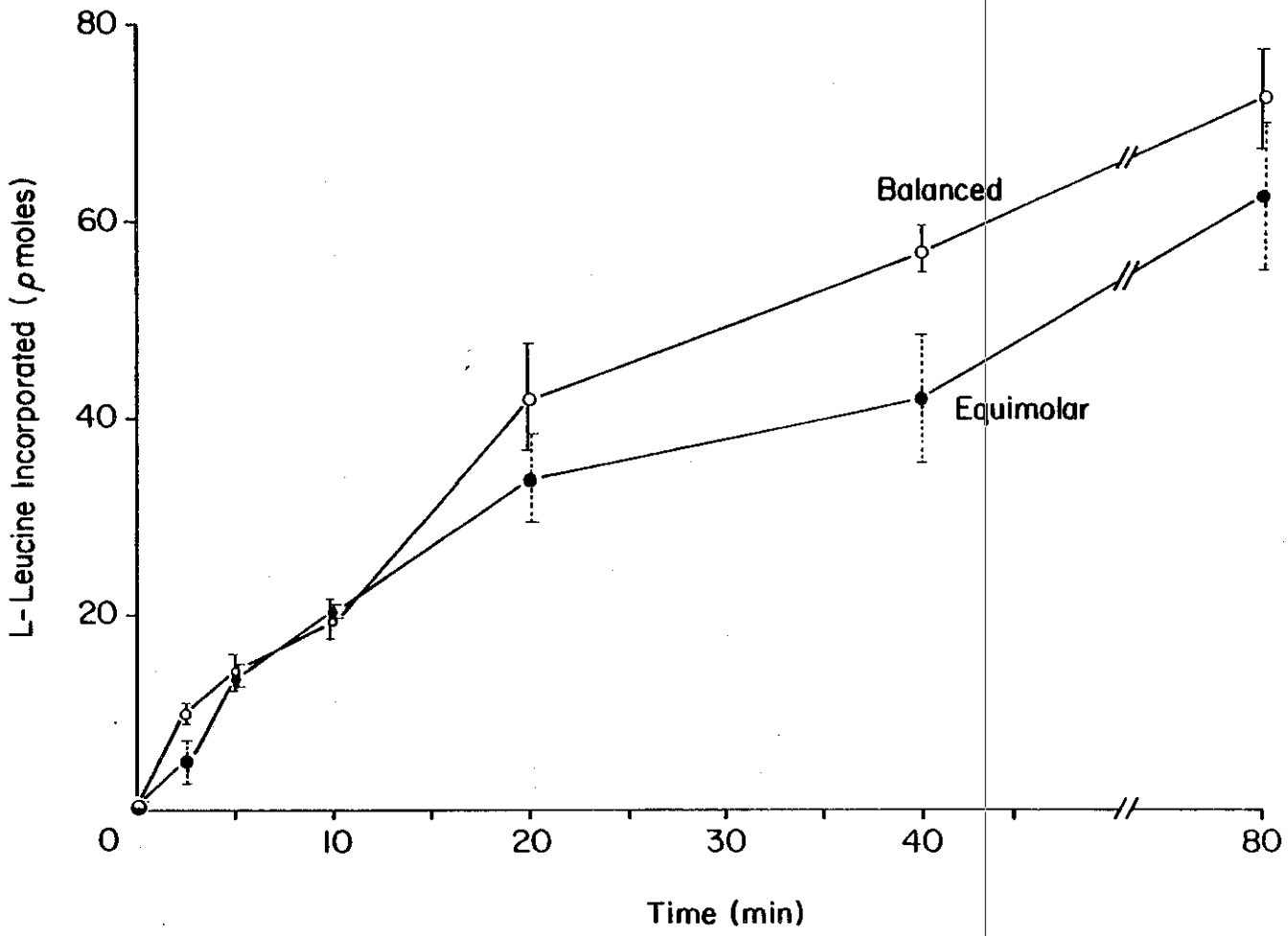

3.0
2.5
2.0
1.5
1.0
0.5
0
25
50
75
Balanced
Equimolar
L-Leucine Incorporated (ρ moles)
Time (min)

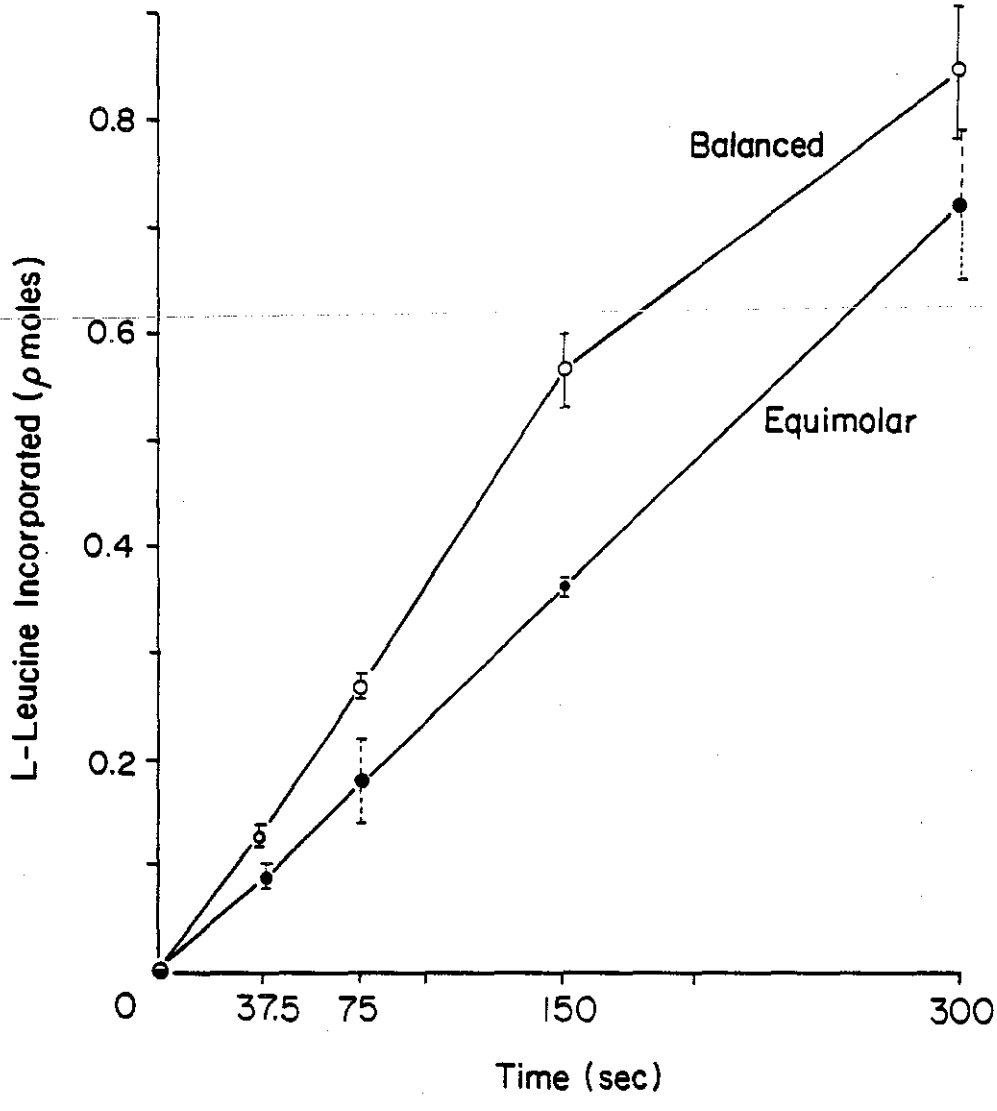
of incorporation in the presence of either a balanced or an equimolar amino acid mixture. The rate of incorporation was greater in the 5 - 20 min. period for the equimolar condition as indicated by the slope, but the extent of incorporation due to changes at earlier times was greatest for the condition where a balanced amino acid mixture was present. Both the rate and extent of incorporation of leucine into protein during the 20 - 40 min. interval was greater when a balanced amino acid mixture was added. However, later time periods showed that the different in the synthesis of protein as a function of the balanced or equimolar amino acid mixtures was decreased.

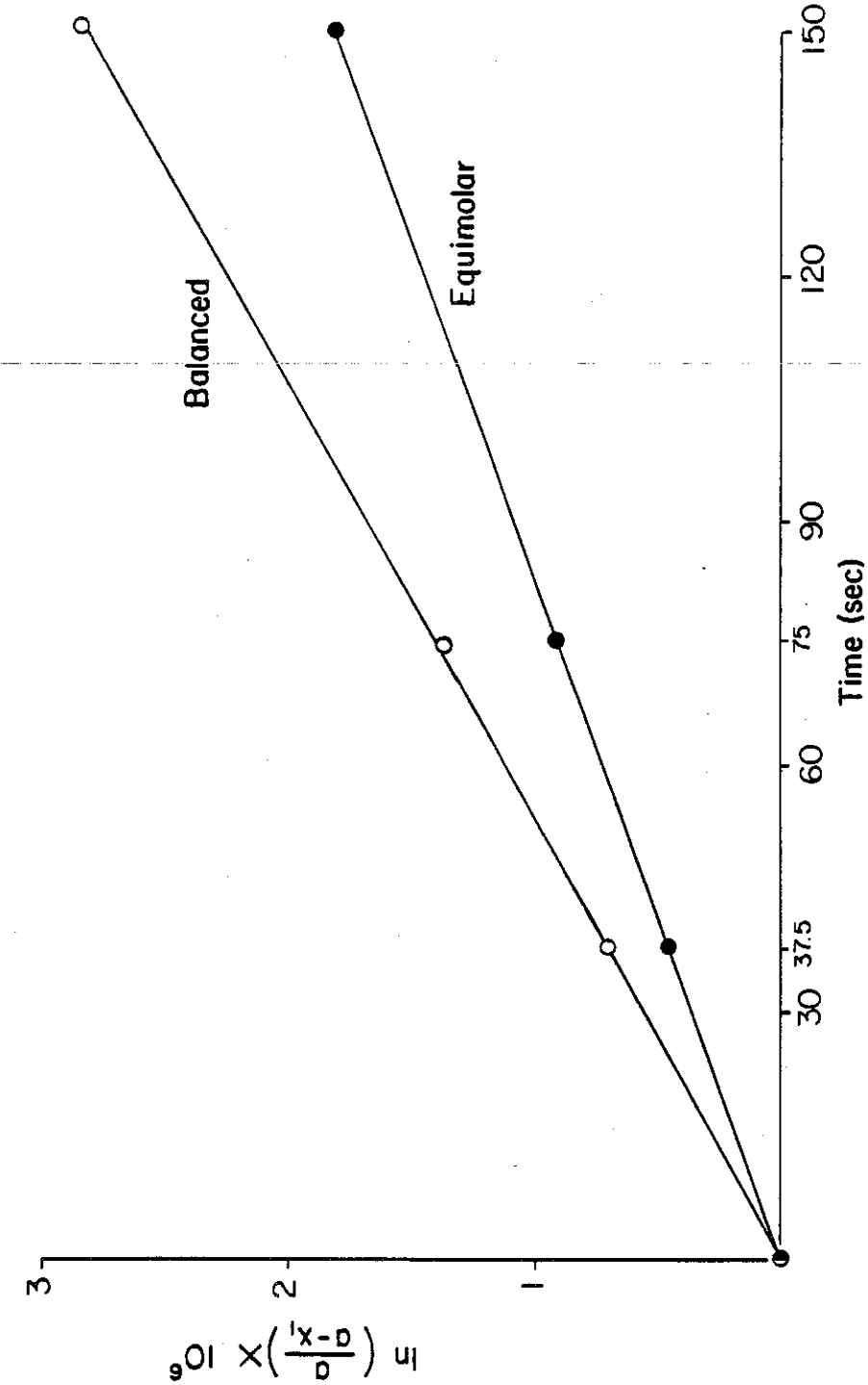
When the leucine concentration was physiological, the differences between the addition of balanced or equimolar amino acid mixtures was not significant, and the incorporation of amino acids into protein was not zero order; the slope was increasing (Figure 22).

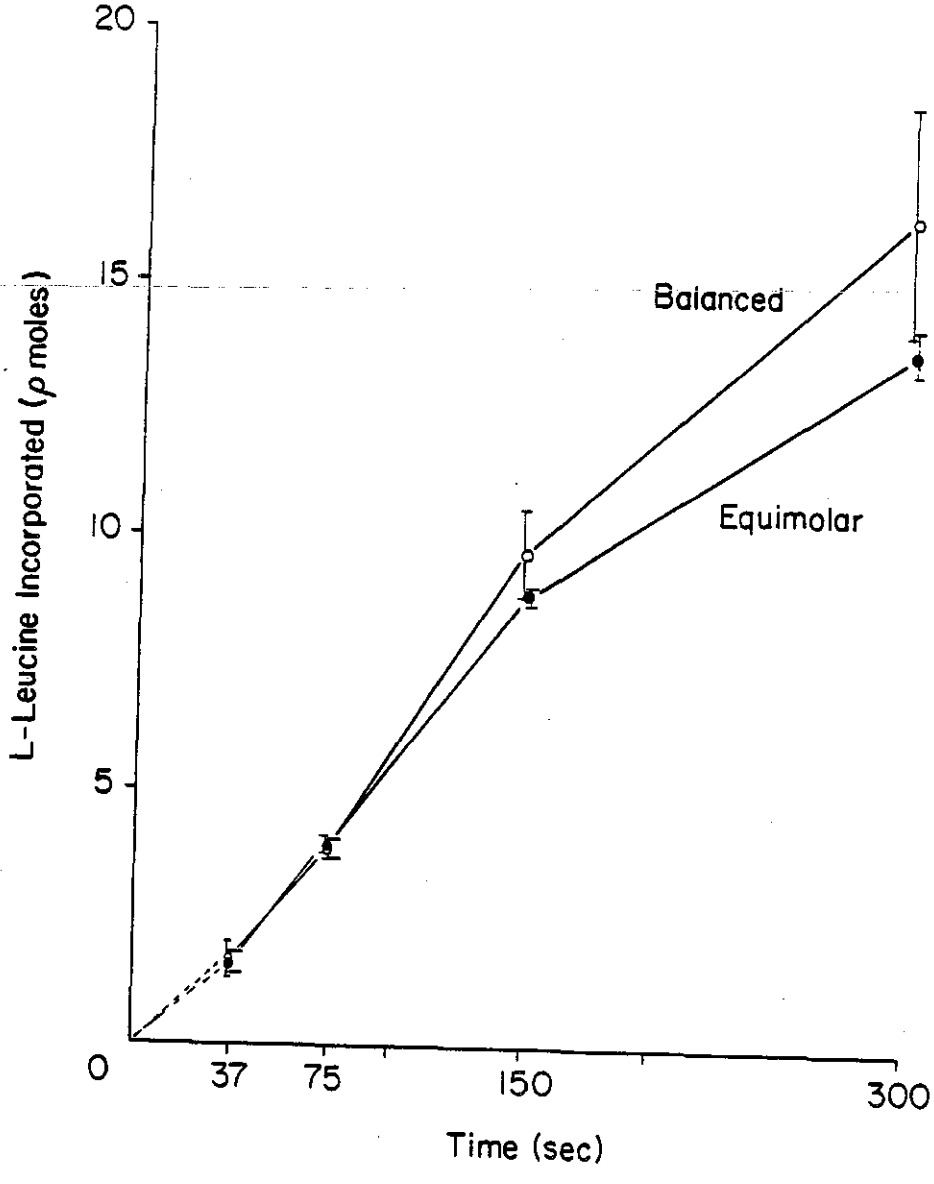
Earlier time periods (37.5 - 300 sec.) indicated that when leucine was limiting, the rate and extent of incorporation into protein differed significantly in the presence of either a balanced or an equimolar amino acid mixture (Figure 23). The kinetics of the reaction were first order, regardless of whether a balanced or equimolar amino acid mixture was added (Figures 23, 24). When leucine was at the physiological concentration of 183 μM, the incorporation during the early time period was not true first order but pseudo-first or mixed order kinetics as shown in Figures 25 through 28. True linearity was maintained only during the time period up to 150 seconds.

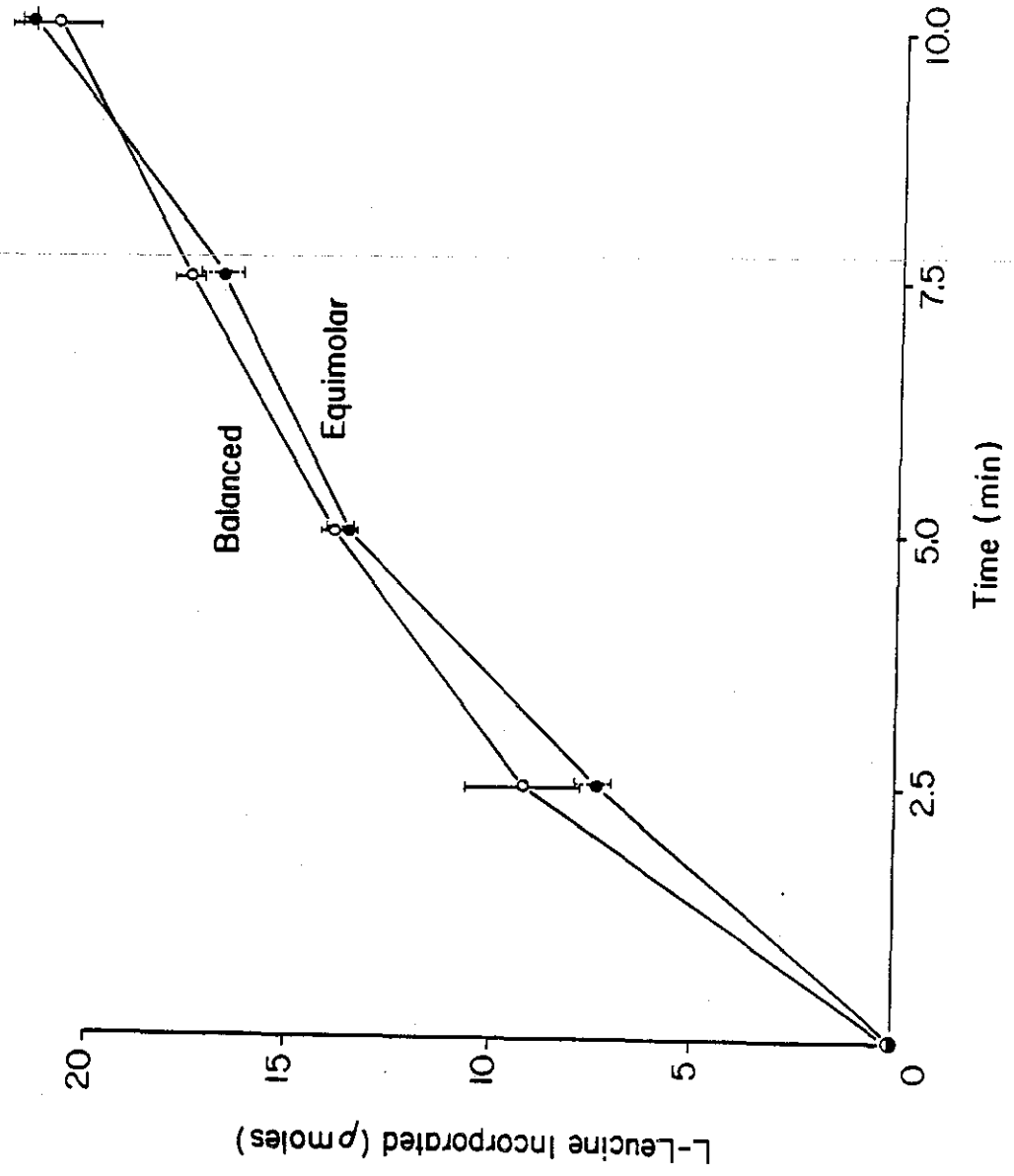
The data based on the rate of incorporation indicates that the only time differences in incorporation occur during the initial stages of the reaction and during the 20 - 40 minute period. The balance of amino acids

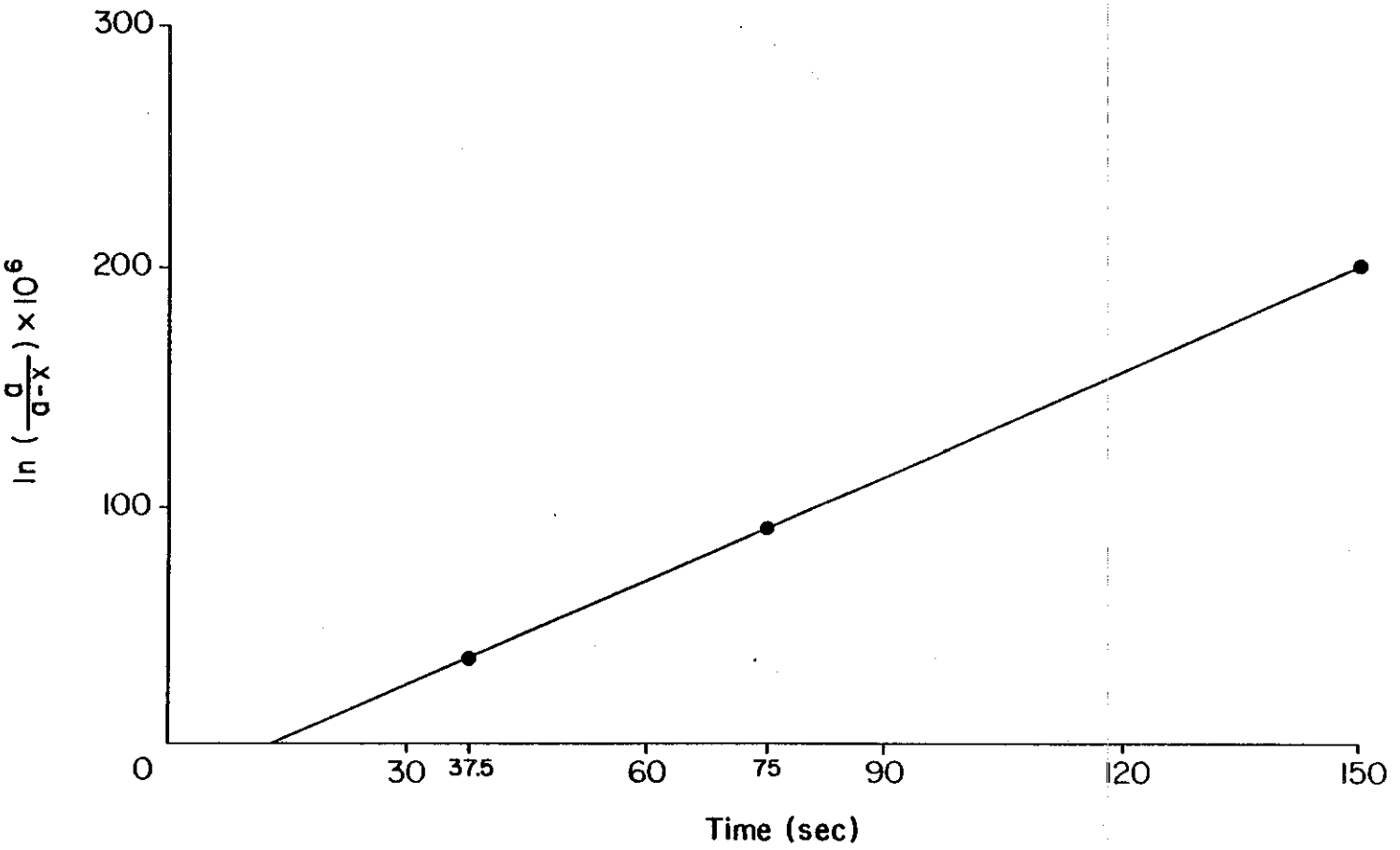

80
60
40
20
0
L-Leucine Incorporated (ρmoles)
Balanced
Equimolar
10
20
30
40
80
Time (min)

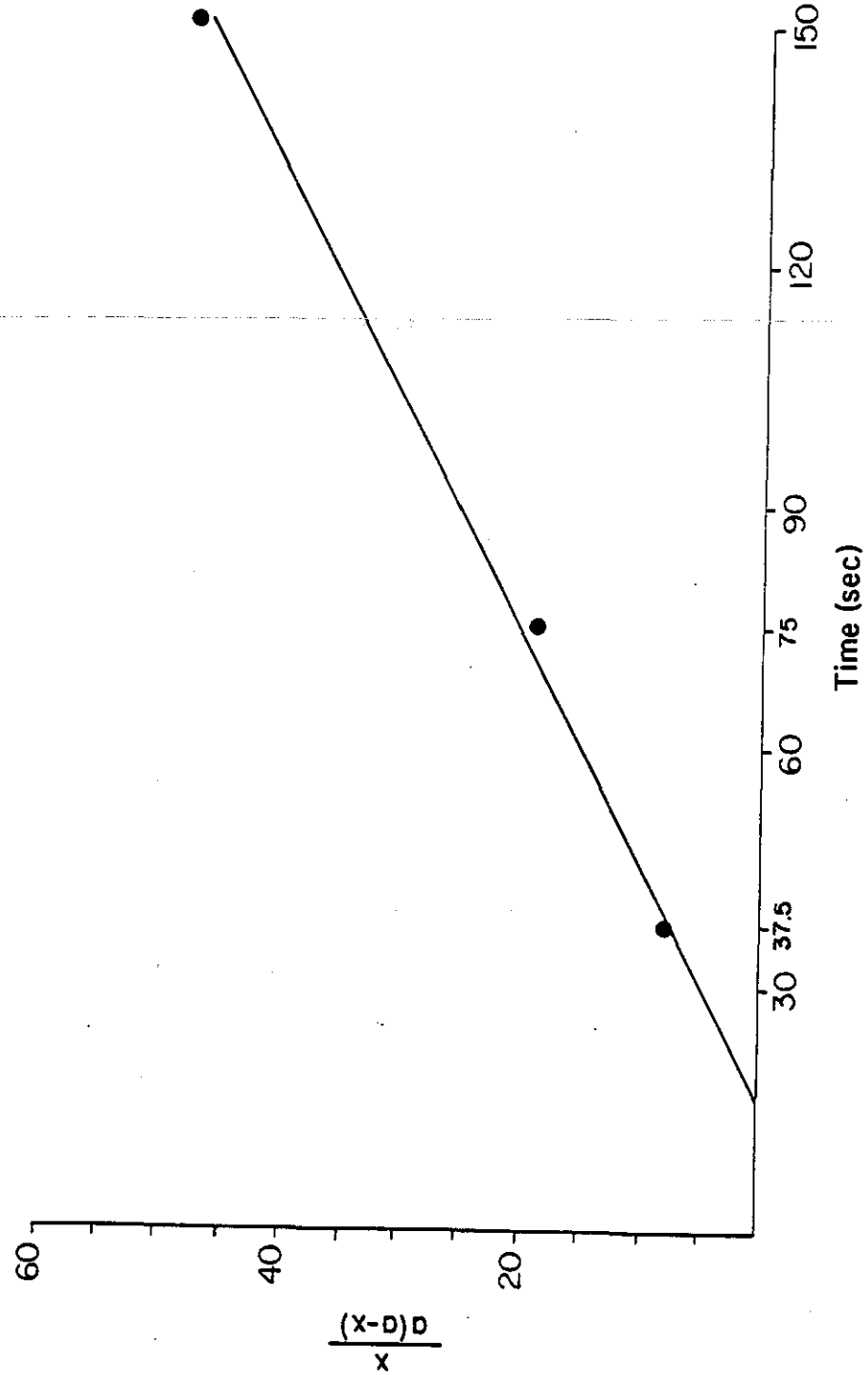

0.8
0.6
0.4
0.2
0
37.5
75
150
300
Balanced
Equimolar
L-Leucine Incorporated (ρ moles)
Time (sec)


Balanced
Equimolar
Time (sec)
30
37.5
60
75
90
120
150
1
2
3
ln (a/(a-x)) x 10^6


20
15
10
5
0
37
75
150
300
Time (sec)
L-Leucine Incorporated (ρ moles)
Balanced
Equimolar


L-Leucine Incorporated (pmoles)
20
15
10
5
0
2.5
5.0
7.5
10.0
Time (min)
Balanced
Equimolar


300
200
100
0
30
37.5
60
75
90
120
150
Time (sec)
ln (a/(a-x)) x 10^6


60
40
20
$\frac{D(D-X)}{X}$
30
37.5
60
75
90
120
150
Time (sec)

seems to protect the system against the limitation of leucine but is not as important as the concentration of leucine.

When the effect of leucine concentration was compared in the presence of a balanced amino acid mixture, the extent of incorporation and time dependency are clearly seen (Fig. 29). The extent of incorporation beyond the 40 min. period was unchanged if leucine was limiting; it was still increasing even at 80 min. if leucine was physiological.

Kinetic Studies of the Leucine Concentration Effect on Protein Syntehsis

The kinetics of protein synthesis as a function of leucine concentration are presented in Figure 30. The supernatant possibly contained two components sensitive to leucine; the reciprocal plot of velocity versus leucine concentration had two distinct regions that could be fitted with different straight lines. The Km and Vmax values, obtained by weighted regression analysis of the two component system are presented in Table 17. There appears to be a low Km (6.4 μM), low Vmax (2.44 x 10^{-9} moles$\cdot l^{-1}\cdot min^{-1}$) and a high Km (114 μM), high Vmax (3.86 x 10^{-9}, moles$\cdot^{-1}\cdot min^{-1}$) component present in the supernatant. The high Km (114 μM) component is within the physiological concentration range for leucine (119 μM) which is discussed in subsequent sections.

For the limiting condition, the first order rate constants derived from the Lineweaver-Burke estimates for Km and Vmax (Table 17; Fig. 30, line B) are fair approximations to those results obtained from the rate studies and rate equations as shown in Table 18. The condition where leucine was physiological could not be evaluated as to reaction order or accuracy of the rate constant method. The rate of incorporation at early time (37.5 - 150 sec.) i.e., the first order portion of the curve,

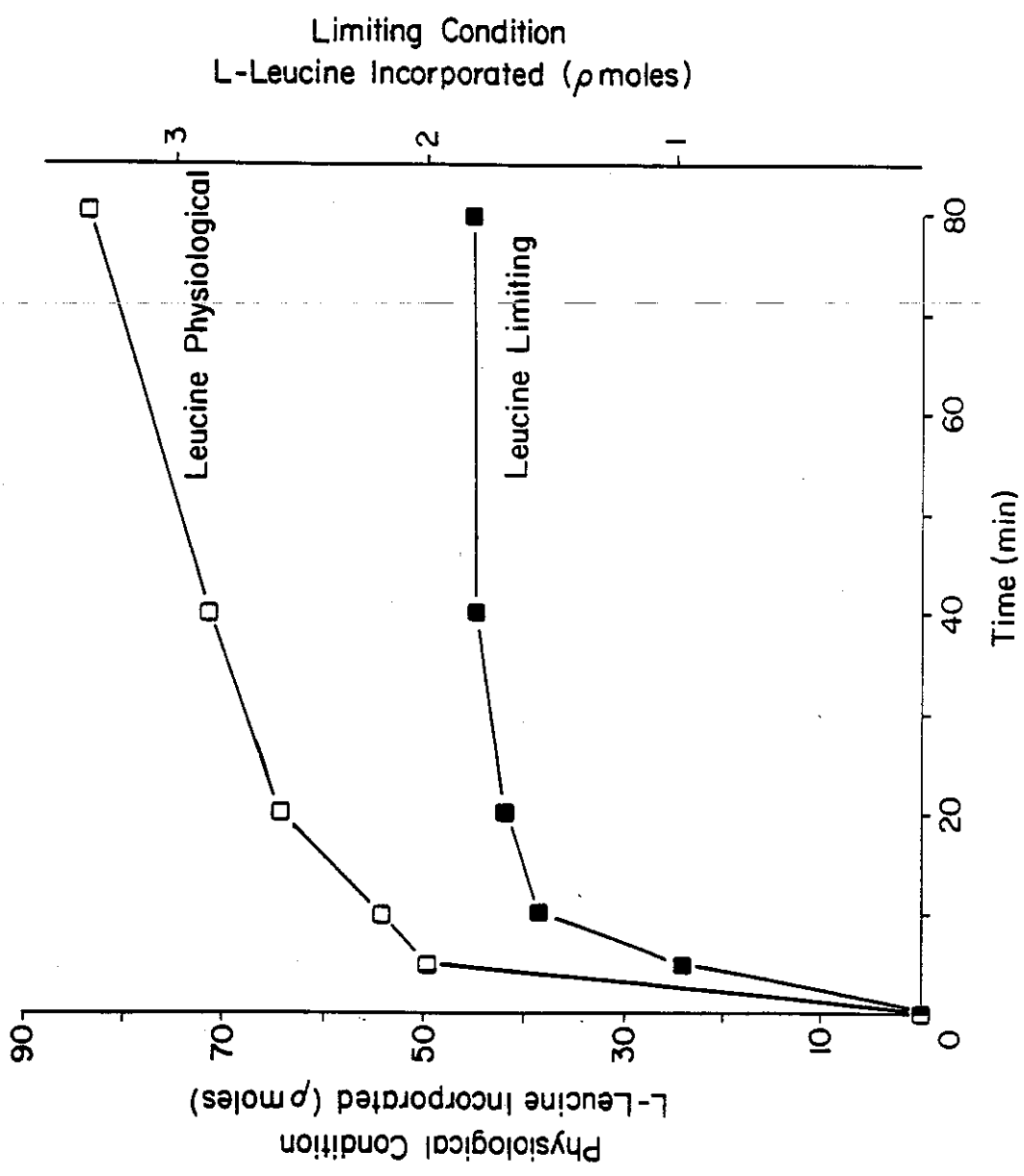

Limiting Condition
L-Leucine Incorporated (ρ moles)
3
2
1
Leucine Physiological
Leucine Limiting
Physiological Condition
L-Leucine Incorporated (ρ moles)
90
70
50
30
10
0
20
40
60
80
Time (min)

Table 17

Michaelis Constants for Liver Protein Synthesis as a Function of Leucine Concentration[1]

Leucine Concentration Range (μM)	Km (μM)	Vmax (10^{-9} moles · L^{-1} · min^{-1})
111 - 265	113.96 ± 0.76	3.86 ± 0.03
0.8 - 111	6.4 ± 1.40	2.44 ± 0.54

[1] Conditions for protein synthesis were as stated in Materials and Methods, except that the leucine concentration was varied from 0.8 μM - 265 μM. A balanced mixture of amino acids was present in the amino acid mix. Incubation time, 5 min.

[2] Provisional estimates of Km and Vmax were calculated by using a weighted regression analysis program (55) based on the derivation of Wilkinson (56).

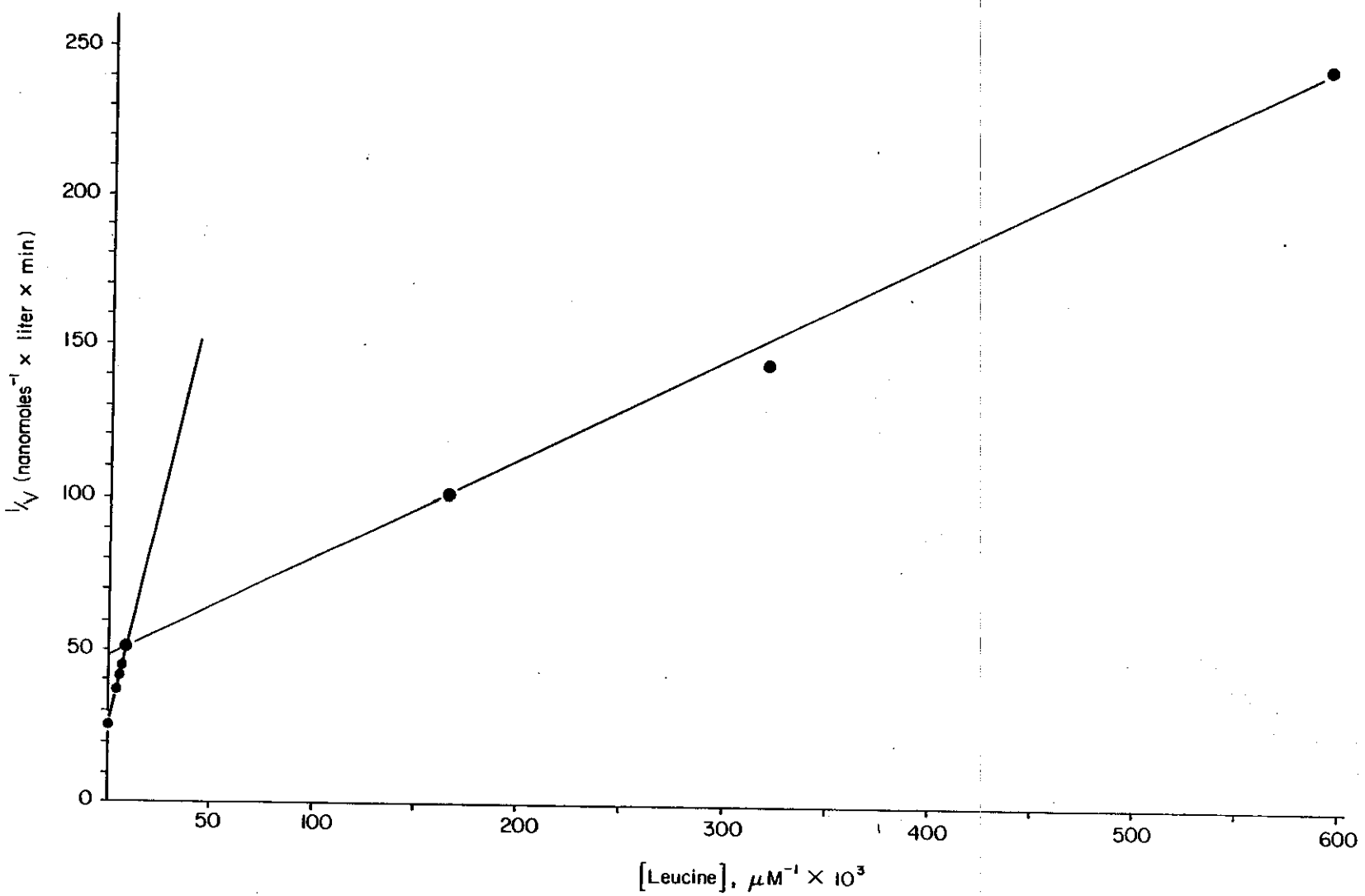

250
200
150
100
50
0
1/V (nanomoles⁻¹ × liter × min)
50
100
200
300
400
500
600
[Leucine], μM⁻¹ × 10³

Table 18

First Order Rate Constant Estimates for Liver Protein Synthesis as a Function of Leucine Limitation

Experimental Condition	k' Michaelis Constant Estimate	k' First Order Rate Equation Estimate
	$10^{-6} \cdot sec^{-1}$	$10^{-6} \cdot sec^{-1}$
Balanced Amino Acids Added	6.3	19.66
Equimolar Amino Acids Added	ND[2]	12.24

[1] Results are based on mean values from duplicates of two separate assays.

[2] Not determined.

seems to be greater when limiting values of leucine are present, but tapers off rather rapidly in comparison to the condition where leucine is physiological.

The Effect of Leucine and Tryptophan on Polysome Distribution

The distribution of the polysome fraction was altered primarily by the concentration of leucine as shown in Table 19. When the concentration of leucine was limiting and the tryptophan concentration was varied, no change in polysome distribution could be observed. However, when the leucine concentration was maintained at a physiological level and the tryptophan concentration changed, the polysome distribution also was altered, as indicated by differences in sedimentation analysis (Fig. 31). These higher molecular weight complexes were observed just by changing leucine concentration. The greatest change occurred when both leucine and tryptophan were physiological. These results indicate that leucine may play a permissive role in polysome distribution, allowing the tryptophan effect to occur. If the leucine concentration becomes limiting, tryptophan cannot alter polysome size.

The Effect of Added Supernatant Concentration on Protein Synthesis as a Function of Leucine Concentration

When leucine was limiting, variation of the supernatant concentration showed that inhibition occurred at higher concentrations of added supernatant (Fig. 32). However, if leucine was present at a physiological level, the increase in supernatant concentration led to an increase of the incorporation of amino acids into protein; no inhibition was observed when up to 3500 µg of protein were added (Fig. 33). This effect was independent of the relative concentration of other amino

Table 19

Polysome Aggregation as a Function of Leucine and Tryptophan Concentration[1,2]

Experimental Condition	S_{obs}[3,4]
Leucine Limiting	
+2 μM Tryptophan	71.5 + 1.10 (2)
+124 μM Tryptophan	72.7 + 0.73 (2)
Leucine Physiological	
+2 μM Tryptophan	ND
+124 μM Tryptophan	195 (1)

[1] The components of the incubation mixture and reaction conditions are described in the Materials and Methods Section. An aliquot of the reaction mixture was characterized by analytical ultracentrifugation in a Spinco model E ultracentrifuge using sedimentation velocity methods for analysis.

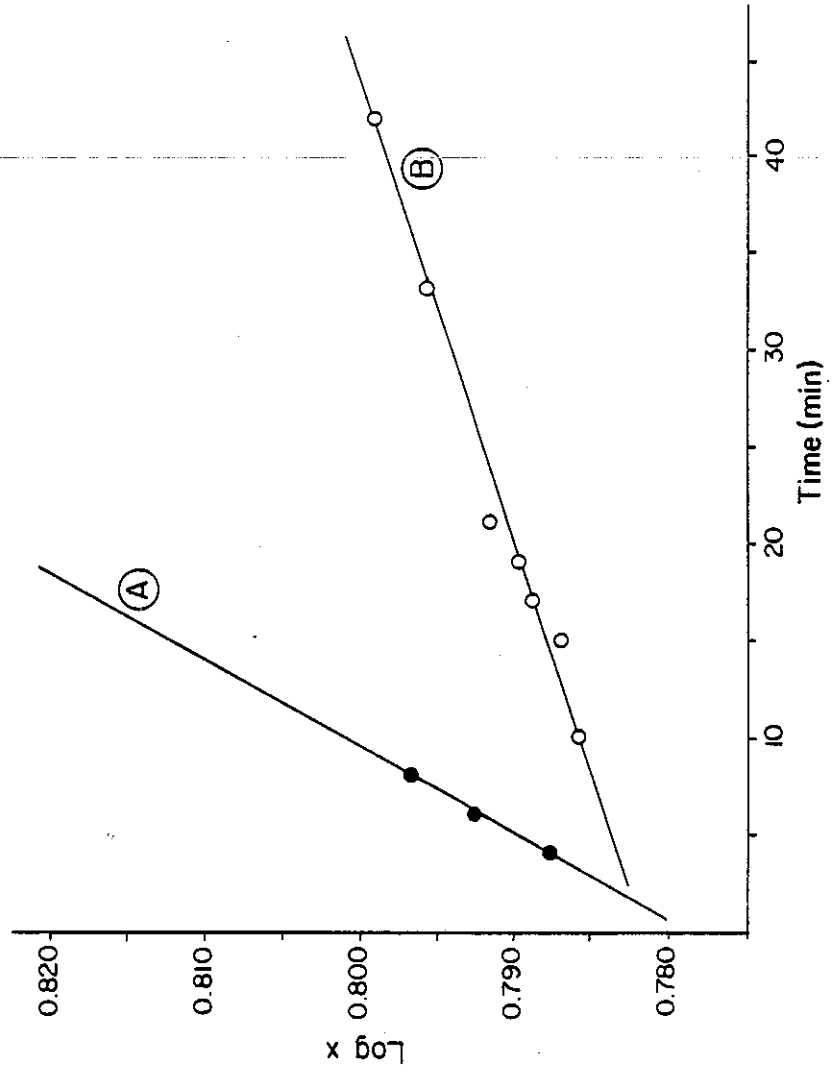
[2] The centrifuge speed was 42,040 rpm for limiting leucine conditions and 15,000 rpm for the leucine physiological conditions. Time intervals between photographs are indicated in Figure 31.

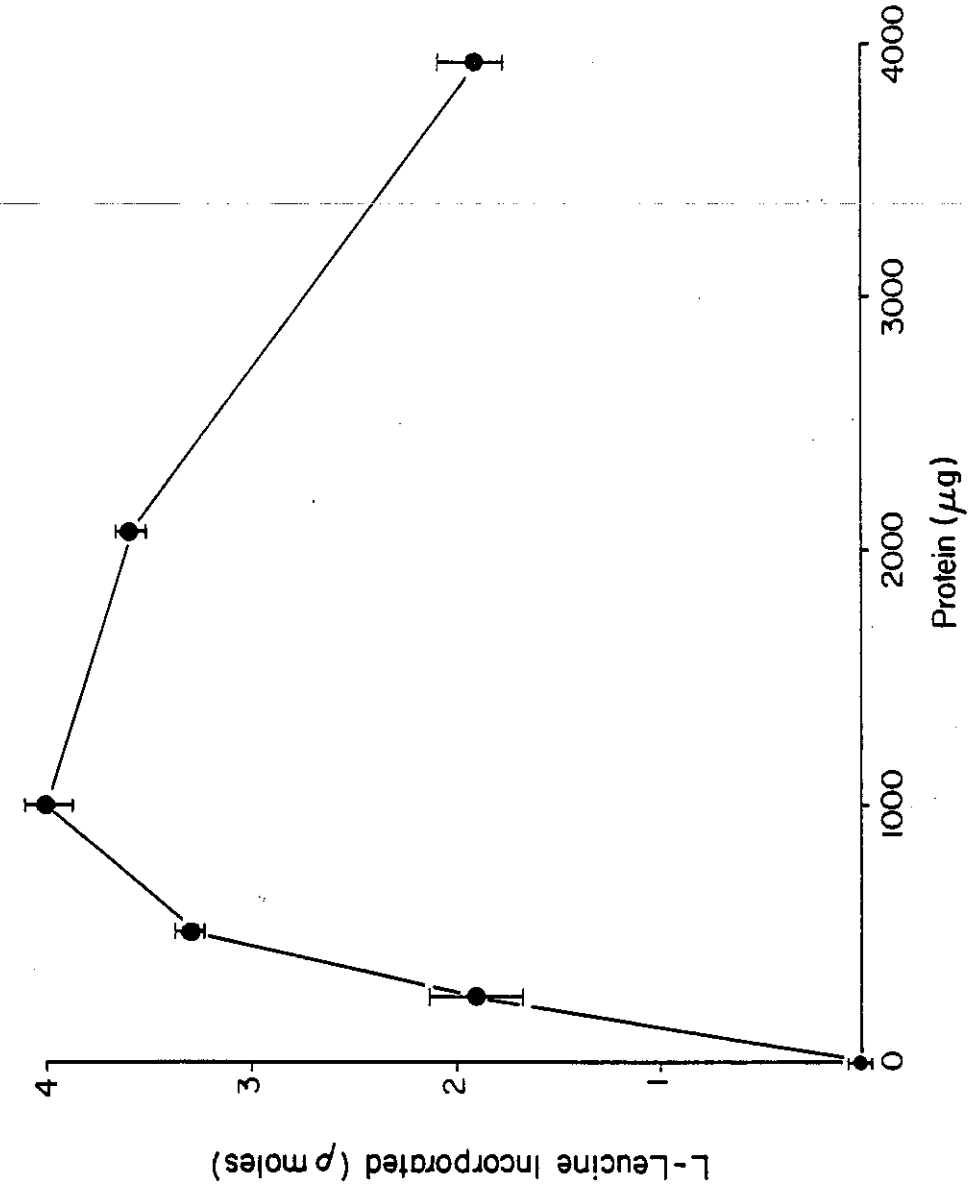
[3] $$S_{obs} = \frac{2.303\ (d \log x/dt)}{(60)(\omega^2)}$$

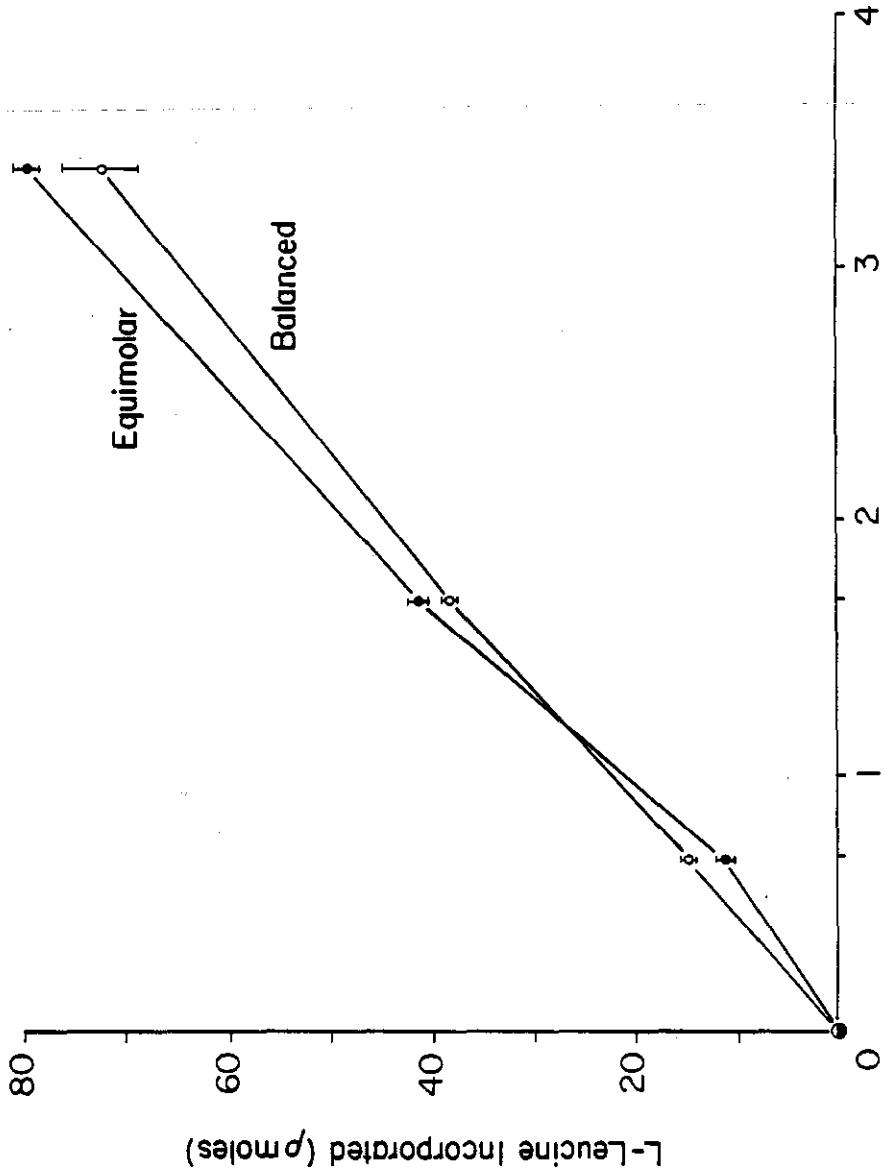
$$= (m;\ \text{slope of log x vs t})\ (R_i,\ \text{Run constant})$$

$$R_c = \frac{(2.303)}{(60)} - (2\pi)\ \frac{(\text{Revolutions} \cdot \text{min}^{-1} \cdot \text{rpm})^2}{(60)}$$

[4] Results are presented as the mean ± standard deviation when n = 2, and as single S_{obs} value when n = 1.


Log x
0.820
0.810
0.800
0.790
0.780
10
20
30
40
Time (min)
A
B


L-Leucine Incorporated (p moles)
4
3
2
1
0
1000
2000
3000
4000
Protein (μg)


L-Leucine Incorporated (p moles)
80
60
40
20
0
1
2
3
4
Protein (μg x 10³)
Equimolar
Balanced

acids. When less than 1000 µg of protein were added, the influence of amino acid balance on protein synthesis is indicated by the velocity curves shown in Figure 34. There was a significant lag in the initial velocity with leucine limiting as cell extract concentration was varied; the response was signoidal. However, if leucine concentration was physiological, an initial linear response to added cell extract was observed.

Removal of an Inhibitor from the 10,000 x g Supernatant Fraction

The preceding experiments demonstrated that under conditions where leucine was limiting an inhibition of protein synthesis occurred with increasing amounts of 10 K-S fraction added. The cell sap was filtered through a Sephadex G-25 column and assayed as described previously. The results indicated that the 10 K-S fraction contained a low molecular weight inhibitor which was removed by treatment with G-25 (Table 20). However, the inhibitor was not completely removed as evidenced by the diminished activity at the higher concentration of added supernatant. The inhibition, when protein concentration increased, after G-25 treatment was not as great if no further addition of amino acids occurred (approximately 32% after correction for activity·mg^{-1} protein). When a balanced mixture of amino acids was present, a 51% inhibition of synthesis was observed at the higher supernatant concentration.

In comparison, dialysis of the 10,000 x g supernatant led to a decrease in protein synthesis regardless of whether amino acids were added in an equimolar or balanced mixture of amino acids (Table 21). The condition for addition of equimolar amino acids was the same as if no additional amino acids were added (not shown). Whereas gel filtration

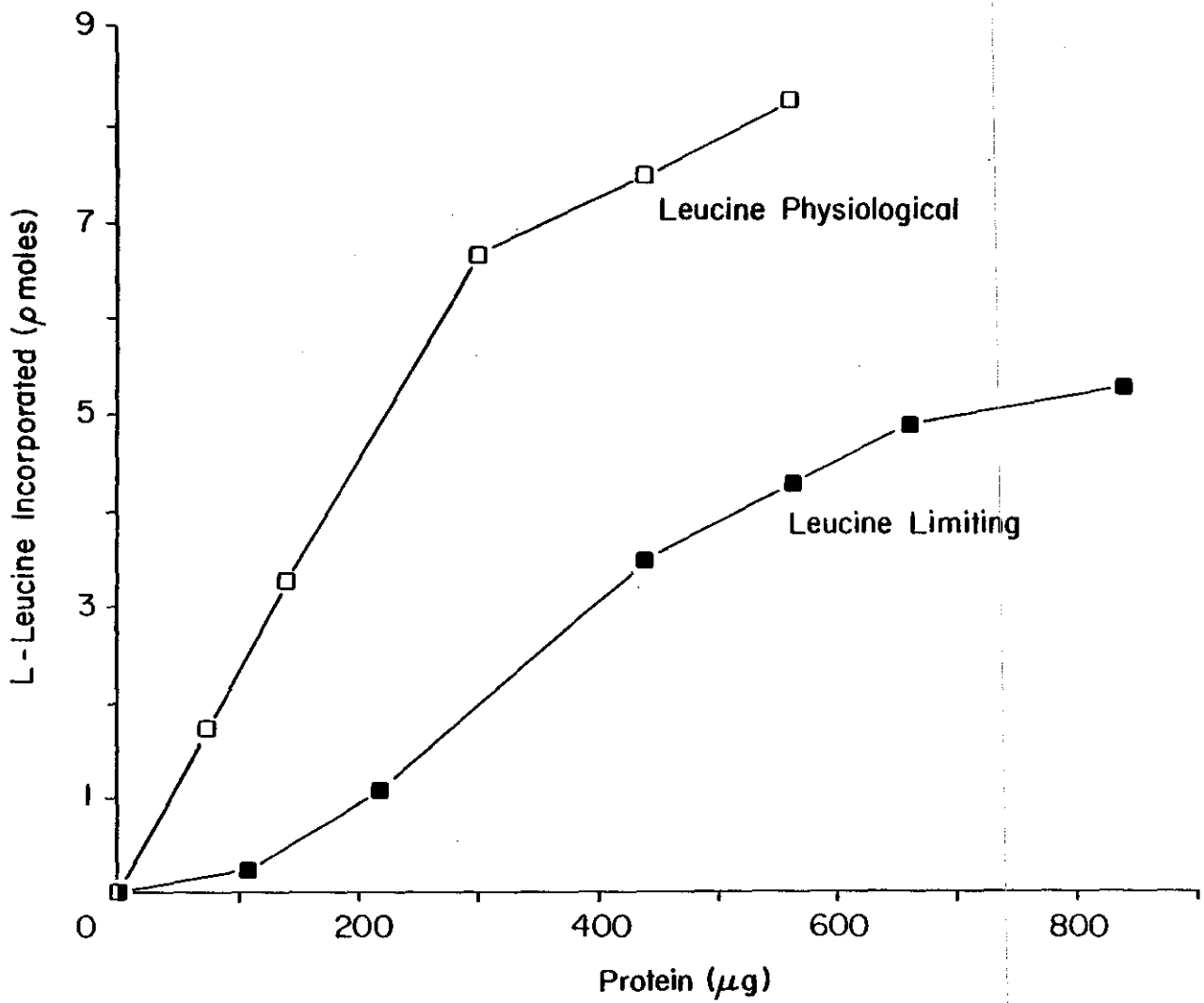

9
7
5
3
1
0
200
400
600
800
L - Leucine Incorporated (ρ moles)
Protein (μg)
Leucine Physiological
Leucine Limiting

Table 20

The Effect of Sephadex G-25 Filtration on the 10,000 x G Supernatant Fraction[1,2]

Experimental Condition	µg Protein [3]	P Moles Leucine Incorporated [4,5]	
Control: (10 K-S)			
No Amino Acids Added	1024	1.86	0.02
+ Balanced Amino Acids	1024	4.93	0.02
G-25 Sephadex Treated:			
No Amino Acids Added	720	7.75	0.41
	1024	7.45	0.23
+ Balanced Amino Acids	720	23.93	0.65
	1024	16.69	0.38

[1] Column, 24 x 1.8 cm, Sephadex G-25; mobile phase, 250 mM sucrose, 25 mM KCl, 8 mM $MgCl_2$, 100 mM Tris-Cl, pH 7.4 at 4°C, and 5 mM β-mercaptoethanol. Vo = 13 ml, Vt = 38 ml, flow rate, 0.46 ml·min^{-1}. Sample size, 1.0 ml.

[2] The details concerning the composition of the reaction mixture and assay for protein synthesis are presented in Materials and Methods.

[3] Micrograms of protein added to incubation mixture.

[4] The concentration of leucine in this experiment was 5 µM, i.e., leucine limiting.

[5] Results are based on mean values ± standard deviation, in two experiments.

Table 21

Loss of Activity by Dialysis of 10 K-S Fraction[1]

Experimental Condition	CPM · mg^{-1} Protein + Balanced Amino Acids	CPM · mg^{-1} Protein + Equimolar Amino Acids
Untreated	89,962	72,223
Dialyzed	15,810	12,708

[1] Liver cell sap, 10 K-S, was dialyzed against 200 volumes of Medium Y; 250 mM sucrose, 25 mM K^+, 8 mM Mg^{2+}, 100 mM Tris-Cl, pH 7.4 at 4°C, and 5 mM β-mercaptoethanol. The protein concentration was then determined. The reaction mixture and incubation conditions are as described in Materials and Methods. The leucine concentration was limiting (5 μM). Results are presented as the average of duplicates from a single experiment.

removed an inhibitor, dialysis most likely removed an essential component of the system. The molecular weight cut-off for the G-25 Sephadex is approximately 100 - 2,000 daltons, whereas the dialysis tubing is greater than 12,000 daltons (Spectrapor-2, Spectrum Medical Industries, Los Angeles, CA).

Effect of Cyclic Nucleotides on Protein Synthesis

Considerable interest has recently been focused on the role of the cyclic nucleotides on protein synthesis. For this reason, the effect of cAMP on protein synthesis as a function of the relative concentration of amino acids was studied (Table 22). No significant differences in protein synthesis were observed with respect to changes in cAMP concentration, especially when related to physiological values. No significant differences were observed with respect to leucine incorporated into the albumin fraction. However, the addition of 10 μM theophylline, a phosphodiesterase inhibitor, depressed total protein synthesis, but stimulated synthesis of the albumin fraction, especially at lower concentrations of cAMP (i.e., 0 - 10 μM). The significance of these findings has not been determined. However it does imply that a concentration dependent effect of cAMP on protein synthesis does exist.

Effect of ATP Concentration on Protein Synthesis

Several investigators have observed an ATP dependent inhibition on protein synthesis (57,58). This effect has been thought to be related to a stimulation of catabolic processes involving activation of lysosomal proteases (58). This effect has not been reproducible (57). However, in these experiments a definite decrease in leucine incorporation into protein was observed as ATP increased above 1 μM (Table 23).

Table 22

Effect of cAMP and Theophylline on Protein Synthesis in the Presence of a Balanced or an Equimolar Amino Acid Mixture[1]

Experimental Condition[2] cAMP (μM)	Incorporation into Total Protein p mole·mg^{-1} protein		Incorporation into Albumin % Albumin Fraction[3]	
	+ Balanced Amino Acid Mixture	+ Equimolar Amino Acid Mixture	+ Balanced Amino Acid Mixture	+ Equimolar Amino Acid Mixture
0	4.93	1.86	2.69	1.62
1	5.02	1.85	2.48	2.53
10	4.70	1.86	2.65	2.90
100	3.99	1.71	3.49	2.47
1000	3.59	1.35	2.92	2.46
+ 10 μM Theophylline				
0	4.32	1.35	3.85	4.30
1	4.50	1.65	3.52	3.40
10	4.45	1.72	3.47	3.23
100	4.12	1.51	4.12	3.30
1000	3.80	1.63	3.00	2.82

[1] The reaction mixture and incubation conditions are as described in Materials and Methods. The leucine concentration was limiting at 1 μM.

[2] Both cAMP and theophylline were prepared in 50 mM Tris-Cl, pH 7.4 at 22°C.

[3] Results are based on single experiment.

Table 23

Effect of ATP Concentration on Protein Synthesis by a Cell-Free System (10 K-S) from Liver[1]

ATP Concentration (mM)	Leucine Incorporated (% of Control Value)[3]
0.5	59
1.0	100
1.5	83
2.0	68
2.5	67
3.0	57
3.5	43
4.0	38
4.5	31
5.0	15

[1] Reaction mix and incubation conditions are the same as described in Materials and Methods.

[2] The amount of leucine incorporated and presented as % is based on cpm·mg^{-1} 10 K-S added.

[3] Results are expressed as average values from two separate experiments.

The results were quite reproducible from one supernatant preparation to another and were independent of leucine concentration or amino acid concentration. A sharp optimum occurs at 1 mM, which represents the physiological concentration. Whether this apparent inhibition is related to depressing the level of synthesis of protein or enhancing degradation has not been determined.

Amino Acid Composition of Plasma from C3HeB/FeJ

The analysis of amino acids from the strain of mice used in these experiments demonstrated that the concentration of specific amino acids changed significantly depending upon whether the animals have been fasted or fed (Table 24). In addition, the amino acid composition of the plasma differed significantly from the balanced mixture derived from published values for "mice" in general. The greatest differences were observed for glutamic (3.2-fold) and arginine (2.7-fold). Most free amino acid concentrations were greater for the condition where animals were starved 12 h. This would be representative of protein degradation and increased amino acid mobilization for gluconeogenesis or changes in plasma volume due to a dehydration effect. The greatest concentration variation observed was for threonine (approximately 2.98-fold). Two amino acids, however, varied inversely and were present at higher concentrations in the plasma from fed animals (Histidine, 1.3-fold; Aspartate, 1.4-fold). The most significant observation was that the concentrations of tryptophan and leucine remained relatively constant regardless of whether the animals were fed or fasted. The concentration of tryptophan was 67 μM; leucine was 119 μM. The Km value for leucine was determined to be 113 μM.

Table 24

Free Amino Acid Composition of Plasma from C3Heb/Fej[1]

Amino Acid	Fed Condition				Fasted Condition			
	mg/100 ml		mM		mg/100 ml		nM	
ARGININE	1.42 ±	0.13	0.077 ±	0.007	2.62 ±	0.08	0.150 ±	0.004
HISTIDINE	1.63	0.01	0.103	0.007	1.25	0.15	0.080	0.010
ISOLEUCINE	1.34	0.04	0.102	0.003	2.91	0.07	0.222	0.005
LEUCINE	1.70	0.15	0.128	0.01	1.66	0.00	0.126	0.001
LYSINE	1.00	0.00	0.109	0.001	5.45	0.36	0.298	0.019
METHIONINE	0.68	0.03	0.045	0.002	1.14	0.13	0.076	0.008
PHENYLALANINE	0.48	0.03	0.029	0.002	1.29	0.17	0.078	0.010
THREONINE	0.87	0.07	0.073	0.005	2.59	0.14	0.218	0.012
TRYPTOPHAN	1.30	0.08	0.066	0.004	1.40	0.09	0.068	0.004
VALINE	2.39	0.44	0.204	0.038	3.38	0.05	0.288	0.005
ALANINE	1.68	0.06	0.189	0.007	3.70	0.015	0.415	0.018
ASPARTIC	0.49	0.026	0.037	0.002	0.36	0.026	0.027	0.002
CYSTEINE	0.27	0.02	0.015	0.000	0.56	0.06	0.032	0.003
GLUTAMIC	0.36	0.01	0.025	0.001	1.06	0.06	0.061	0.020
GLUTAMINE	4.76	0.52	0.326	0.035	9.61	0.69	0.657	0.015
GLYCINE	1.01	0.01	0.135	0.002	2.63	0.55	0.350	0.73
SERINE	0.87	0.16	0.083	0.015	2.30	0.33	0.219	0.031
TYROSINE	1.15	0.08	0.063	0.002	1.55	0.29	0.085	0.016

[1] Values for amino acid analysis were obtained from five chromatographic runs for each condition. The mean ± SEM are presented for each amino acid.

Comparison of Plasma Amino Acid Composition of C3HeB/FeJ as a Function of Age

A significant difference in plasma-free amino acid concentrations was observed as a function of age (Table 25.) The leucine and tryptophan plasma concentrations were decreased in animals one month of age. The concentration of leucine did not vary as a function of fasting. The tryptophan value could not be determined in the plasma of the one-month-old, fed animals. However, the concentration of tryptophan in fasted animals was significantly lower than similarly treated animals three months of age.

The non-essential amino acid concentrations were considerably higher at one month than at three months; glutamic was 12.9-fold higher in the plasma of one-month-old, fed animals than in the three-month-old animals. Of the essential amino acids, threonine was six times higher in the one-month-old animals as compared to values at three months of age.

The total free amino acid concentration was not generally higher for plasma of one-month-old, fasted animals in comparison to one-month-old, fed animals.

Amino Acid Concentration of 10 K-S Fraction

The free amino acid concentration of the supernatant was generally greater than the plasma concentration of amino acids (Table 26). Leucine concentration did not change significantly as a function of fasting (0.120 mM, fed; 0.131 mM, fasting), whereas tryptophan did (0.065 mM, fed; 0.024 mM, fasting). The 10 K-S concentration of amino acids was generally greater for fed animals in comparison to starved animals.

Table 25

Age Comparison of the Amino Acid Composition of Plasma from C3HeB/FeJ[1]

	Fed Condition								Fasted Condition							
	1 Mo				3 Mos.				1 Mo.				3 Mos.			
	mg/100 ml		mM		mg/100 ml		mM		mg/100 ml		mM		mg/100 ml		mM	
ARGININE	ND[2]		ND		1.42 ± 0.13		0.077 ± 0.007		0.79 ± 0.00		0.045 ± 0.001		2.26 ± 0.08		0.150 ± 0.004	
HISTIDINE	2.10 ± 0.33		0.134 ± 0.022		1.63	0.01	0.143	0.009	2.23	0.10	0.143	0.005	1.25	0.15	0.080	0.010
ISOLEUCINE	1.31	0.06	0.100	0.005	1.34	0.04	0.102	0.003	1.96	0.26	0.150	0.02	2.91	0.07	0.222	0.005
LEUCINE	1.34	0.11	0.102	0.083	1.70	0.15	0.128	0.01	1.42	0.09	0.108	0.007	1.66	0.00	0.126	0.001
LYSINE	3.80	0.40	0.207	0.021	2.00	0.00	0.109	0.001	3.28	0.05	0.179	0.03	5.45	0.36	0.298	0.019
METHIONINE	0.69	0.03	0.046	0.001	0.68	0.03	0.045	0.002	0.61	0.09	0.408	0.07	1.14	0.13	0.076	0.008
PHENYLALANINE	0.75	0.10	0.045	0.006	0.48	0.03	0.029	0.010	1.06	0.08	0.064	0.004	1.29	0.17	0.078	0.010
THREONINE	5.80	0.04	0.487	0.04	0.87	0.07	0.073	0.005	6.36	0.85	0.534	0.07	2.59	0.14	0.218	0.012
TRYPTOPHAN	ND		ND		1.30	0.08	0.066	0.004	0.395	0.007	0.019	0.001	1.40	0.09	0.068	0.004
VALINE	1.00	0.007	0.085	0.001	2.93	0.44	0.204	0.038	0.126	0.02	1.48	0.29	3.38	0.05	0.288	0.005
ALANINE	6.76	0.71	0.759	0.08	1.68	0.06	0.189	0.007	7.11	0.97	0.798	0.01	3.70	0.015	0.415	0.018
ASPARTIC	2.74	0.28	0.206	0.02	0.49	0.026	0.037	0.002	1.58	0.04	0.127	0.01	0.36	0.026	0.027	0.002
CYSTEINE	0.43	0.07	0.024	0.004	0.27	0.02	0.015	0.000	1.31	0.00	0.074	0.00	0.56	0.06	0.032	0.003
GLUTAMIC	4.66	0.146	0.318	0.01	0.36	0.01	0.025	0.001	5.16	0.20	0.351	0.01	1.06	0.06	0.061	0.020
GLUTAMINE	15.16	4.15	1.03	0.27	4.76	0.52	0.326	0.035	10.55	0.18	0.722	0.12	9.61	0.69	0.657	0.015
GLYCINE	3.03	0.07	0.403	0.009	1.01	0.01	0.135	0.002	4.60	0.15	0.613	0.02	2.63	0.55	0.350	0.073
SERINE	4.06	0.09	0.387	0.009	0.87	0.16	0.083	0.015	4.75	0.27	0.452	0.02	2.30	0.33	0.219	0.031
TYROSINE	0.43	0.002	0.023	0.001	1.15	0.08	0.063	0.002	0.98	0.03	0.054	0.018	1.55	0.29	0.085	0.016

[1] The values were obtained from five separate chromatographic analyses of samples obtained from twelve different animals. The mean ± SEM are presented for each amino acid.

[2] Not detected.

Table 26

Amino Acid Concentration of 10,000 x g (10 K-S) Liver Supernatant[1]

Amino Acid	Fed Condition		Fasted Condition	
	mg/100 ml	mM	mg/100 ml	mM
ARGININE	ND[2]	ND	ND	ND
HISTIDINE	2.46 ± 0.18	0.148 ± 0.020	1.85 ± 0.24	0.119 ± 0.009
ISOLEUCINE	1.07 0.20	0.082 0.010	0.87 0.00	0.066 0.001
LEUCINE	1.58 0.07	0.120 0.005	1.73 0.18	0.131 0.009
LYSINE	2.81 0.52	0.154 0.020	1.95 0.22	0.107 0.010
METHIONINE	0.57 0.009	0.040 0.001	0.73 0.12	0.049 0.015
PHENYLALANINE	0.65 0.12	0.039 0.008	0.59 0.00	0.036 0.001
THREONINE	8.76 1.90	0.740 0.160	3.13 0.56	0.263 0.040
TRYPTOPHAN	1.34 0.19	0.065 0.005	0.50 0.00	0.024v 0.001
VALINE	1.56 0.30	0.130 0.020	0.55 0.13	0.045 0.008
ALANINE	9.77 1.40	1.090 0.150	6.62 0.74	0.743 0.081
ASPARTIC	15.30 1.50	1.150 0.110	7.90 6.60	0.593 0.120
CYSTEINE	0.71 0.10	0.040 0.005	0.83 0.20	0.049 0.011
GLUTAMIC	6.35 0.70	0.430 0.051	5.30 0.94	0.360 0.060
GLUTAMINE	13.05 1.50	0.890 0.100	14.80 1.65	1.01 0.110
GLYCINE	5.98 0.90	0.800 0.120	3.54 0.14	0.479 0.020
SERINE	4.83 0.51	0.460 0.054	2.58 0.49	0.246 0.040
TYROSINE	0.25 0.04	0.013 0.001	ND	ND

[1] The values were obtained from five separate chromatographic analyses of samples obtained from the livers of twelve different animals. The mean ± SEM are presented for each amino acid.

[2] Not detected.

Tyrosine could not be detected in the supernatant of fasted animals. Arginine could not be detected under either set of conditions.

The ratios of amino acids for all of the tested conditions are presented in Table 27. Amino acid chromatograms are shown in Figs. 35 and 36.

DISCUSSION

Studies indicate that leucine is the primary amino acid regulating protein synthesis, regardless of the concentrations of other amino acids including tryptophan. Both quantitative and qualitative control of translation are suggested by the experiments described here. Quantitative regulation, i.e., the control of the overall rate of protein synthesis in cells, could be important in determining tissue size and its response to changing environmental conditions such as nutritional and hormonal states. Qualitative regulation, involving changes in the relative amounts of different proteins synthesized, such as albumin, could be of great importance for the hormonal induction of enzymes and also for changing patterns of gene expression which accompany cell differentiation.

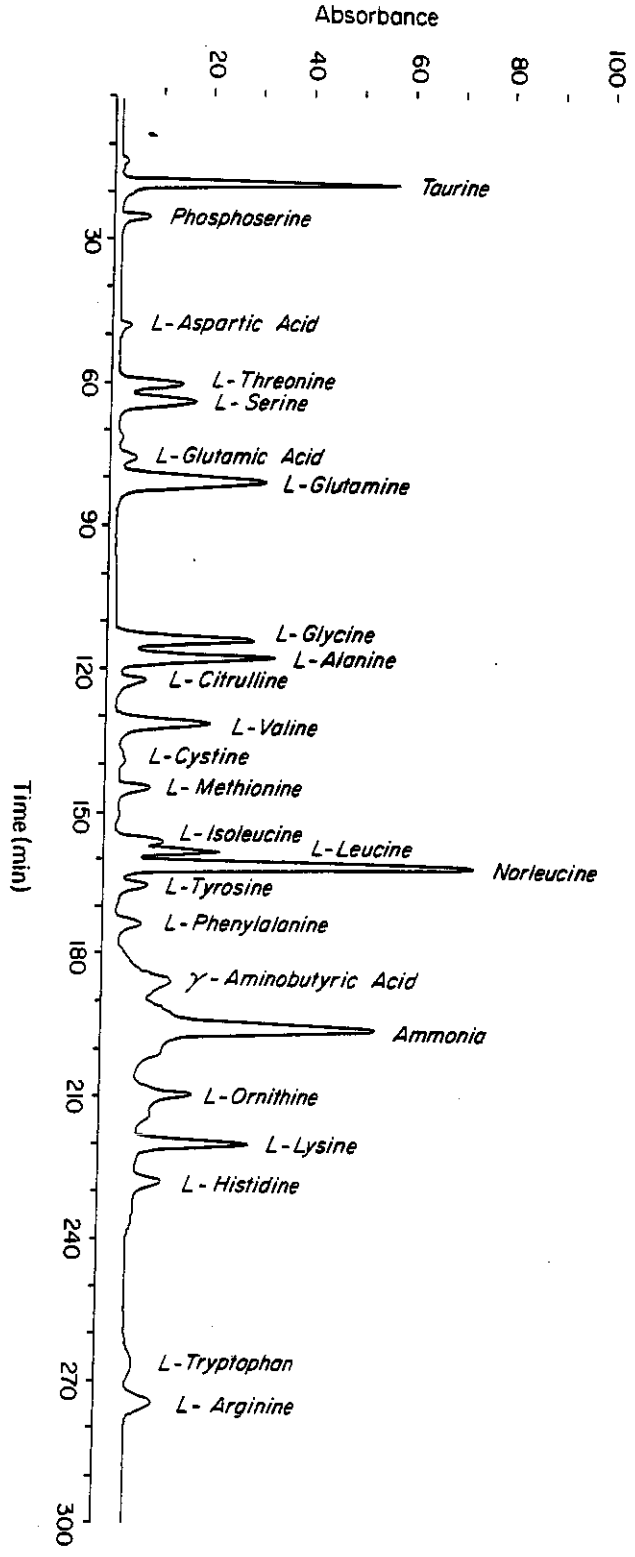
Inhibitory and Stimulating Effects of Leucine on Protein Synthesis

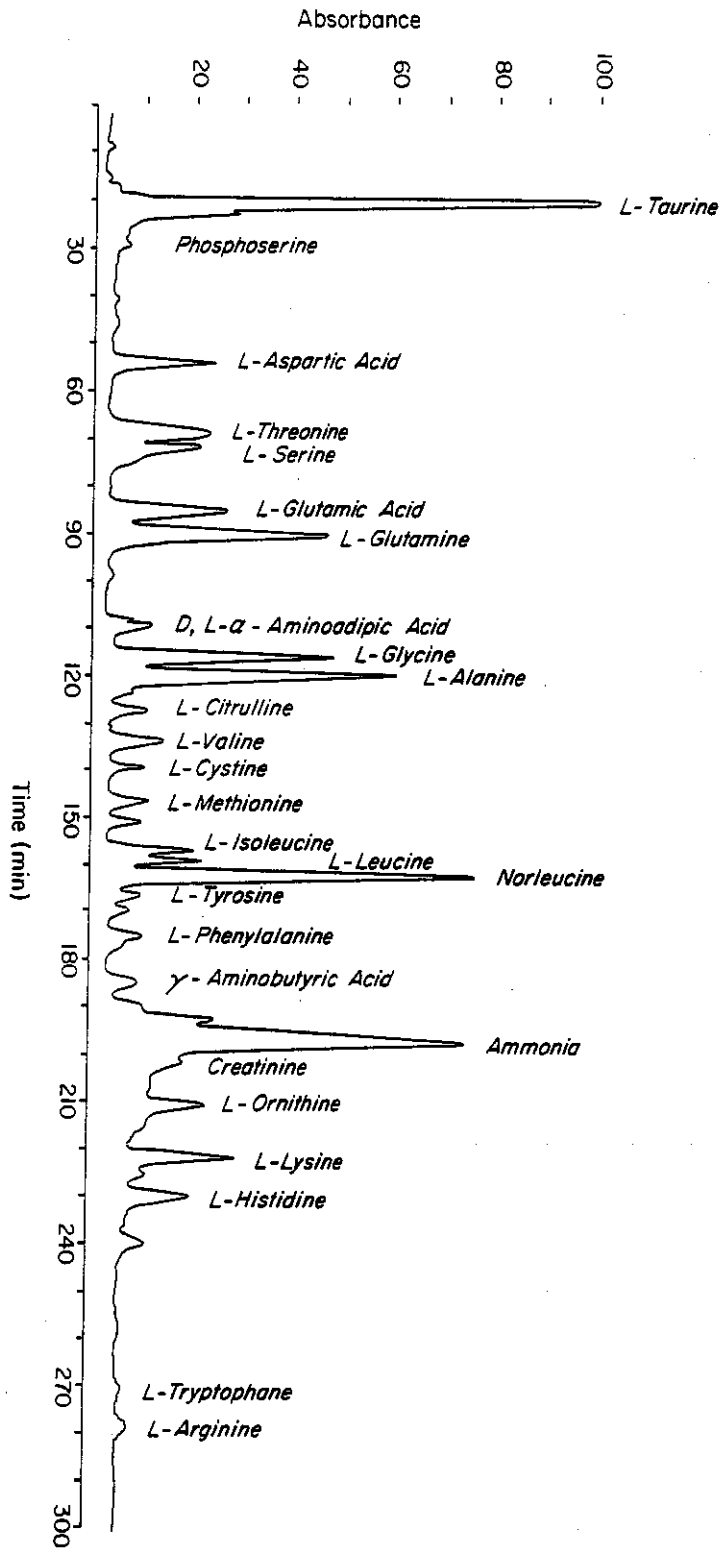
Different results are presented with regard to whether leucine inhibits or stimulates synthesis of protein. The observed leucine inhibition described in this investigation (Fig. 8) is not a new phenomenon. Similar observations were reported by Ibrahim, et al. (12) with respect to rat liver mitochondrial protein synthesis. Inhibition of protein synthesis was observed at concentrations greater than 16 μM. Suskova, et al. (13) demonstrated that leucine, at a concentration of 3 - 12 mM

Table 27

Ratio of Amino Acids

Amino Acid	Plasma 1 Mo.		Plasma 3 Mos.		Supernatant 3 Mos.	
	Fed	Fasted	Fed	Fasted	Fed	Fasted
ARGININE	ND	0.42	0.60	1.19	ND	ND
HISTIDINE	1.31	1.32	1.12	0.63	1.23	0.91
ISOLEUCINE	0.98	1.39	0.79	1.76	0.68	0.50
LEUCINE	1.00	1.00	1.00	1.00	1.00	1.00
LYSINE	2.03	1.66	0.85	2.36	1.28	0.82
METHIONINE	0.45	3.77	0.35	0.60	0.33	0.37
PHENYLALANINE	0.44	0.59	0.22	0.62	0.32	0.27
THREONINE	4.77	4.94	0.57	1.73	6.16	2.01
TRYPTOPHAN	ND	0.17	0.52	0.54	0.54	0.18
VALINE	0.83	13.70	1.59	2.28	1.08	0.34
ALANINE	7.44	7.38	1.48	3.29	9.08	5.67
ASPARTIC	2.02	1.17	0.29	0.21	9.58	4.52
CYSTEINE	0.24	0.69	0.12	0.25	0.33	0.37
GLUTAMIC	3.12	3.25	0.19	0.48	3.58	2.75
GLUTAMINE	10.09	6.68	2.54	5.21	7.42	7.70
GLYCINE	3.95	5.67	1.05	2.77	6.66	3.65
SERINE	3.79	4.18	0.65	1.73	3.83	1.88
TYROSINE	0.22	0.50	0.49	0.67	0.11	ND


Absorbance
20
40
60
80
100
Time (min)
30
60
90
120
150
180
210
240
270
300
Taurine
Phosphoserine
L-Aspartic Acid
L-Threonine
L-Serine
L-Glutamic Acid
L-Glutamine
L-Glycine
L-Alanine
L-Citrulline
L-Valine
L-Cystine
L-Methionine
L-Isoleucine
L-Leucine
Norleucine
L-Tyrosine
L-Phenylalanine
γ-Aminobutyric Acid
Ammonia
L-Ornithine
L-Lysine
L-Histidine
L-Tryptophan
L-Arginine


Absorbance
20
40
60
80
100
Time (min)
30
60
90
120
150
180
210
240
270
300
L-Taurine
Phosphoserine
L-Aspartic Acid
L-Threonine
L-Serine
L-Glutamic Acid
L-Glutamine
D, L-α-Aminoadipic Acid
L-Glycine
L-Alanine
L-Citrulline
L-Valine
L-Cystine
L-Methionine
L-Isoleucine
L-Leucine
Norleucine
L-Tyrosine
L-Phenylalanine
γ-Aminobutyric Acid
Ammonia
Creatinine
L-Ornithine
L-Lysine
L-Histidine
L-Tryptophane
L-Arginine

inhibited the glycyl-tRNA synthetase reaction, thus inhibiting translation (9 - 23%) by limiting the availability of glycine - tRNAgly. This observation was made in a rat liver cell-free protein synthesizing system.

The concentration of leucine that inhibited protein synthesis in our investigation (Fig. 8) was low (18 μM) when compared to the estimated physiological concentration of 183 μM. The microsome to enzyme ratio used in this experiment was not comparable to the in vivo ratio. In addition, the microsome to enzyme fraction was not varied and other dilutions were not tested. The mitochondrial system of Ibrahim, et al. (12) was significantly different from the cytosolic protein synthesizing system. The ribosomal system is a true prokaryotic one with 70S ribosomes; the eukaryotic cytosolic system is composed of 80S ribosomes. The amino acid concentration within the mitochondria has not been determined. The inhibition, however, is observed at a relatively low concentration. In contrast, the concentration of leucine (3 - 12 mM) observed to be inhibitory in the experiments of Suskova, et al. (13) was extremely high and may have no significance at all in terms of a physiological effect.

The studies mentioned above and those of Pitot (9) stressing the importance of ribosome-membrane interaction and finally, the observations reported by Hill, et al. (10), indicated that the experimental conditions used for the study of cell-free protein synthesis were highly unphysiological. For these reasons, the less fractionated 10 K-S system described by Hill, et al. (10) was established as the liver system for use in the continuation of this investigation.

The 10 K-S system was not inhibited by added leucine at concentrations which have been determined to be twice the plasma concentration of approximately 120 μM (Fig. 9). Leucine stimulated protein synthesis when

added to a concentration of 265 μM. This indicated that the previous leucine inhibition at 16 μM and 18 μM was due to experimental conditions. Other investigators have since shown that the branched chain amino acids could enhance phytohemagglutinin activation of lymphocytes in cell culture (30). Tischler, et al. (59) indicated that leucine stimulated synthesis of protein in isolated striated muscle cells. The observed leucine effects and the opposing results obtained with the different liver cell-free preparations suggested that possibly the relative concentrations of other amino acids might be important for protein synthesis.

Design of a Balanced Amino Acid Mixture

The establishment of the components of the balanced amino acid mixture, therefore, became an important prerequisite for this investigation. Plasma amino acid concentrations obtained from literature values for mice (no species or strain designation) were chosen to closely simulate physiological conditions. The steady state concentrations of amino acids found in the systemic plasma (non-portal) of animals have been found to remain relatively constant in spite of wide dietary differences (33). Homeostatic mechanisms seem to control the relative concentrations of individual amino acids in plasma. Any addition or removal of any amino acid for whatever purpose will induce a temporary perturbation which could exert a limiting or regulatory effect on protein synthesis. This implies that rapid equilibria occur between plasma and other body tissues or compartments. These concentrations and relative plasma levels appear to be characteristic of animal species, and suggest that an approach to equilibrium is maintained by the balanced input and removal of amino acids (33). In addition, the average composition of

tissue proteins does not necessarily reflect the pool concentrations of amino acids (33). These findings imply that a relatively stable balance of amino acids is available from the tissue pools for synthesis of proteins under normal conditions in vivo. More recently, several investigators have shown that extracellular amino acids are directly utilized for protein synthesis without mixing with the intracellular amino acid pool (60 - 64). These conclusions were based on experiments which demonstrated linear incorporation of radioactive amino acids into protein before equilibration of the labeled amino acid with the intracellular pool and support the concept of an amino acid mixture based on plasma values.

An imposed experimental alteration of the system used in this investigation was established by limiting the concentration of leucine. This practice has been used widely to maintain high levels of radioactive specific activity for increased sensitivity in detecting synthesis of proteins. The estimated plasma concentration of leucine (approximately 183 μM) is in excess of the initial conditions established (approximately 5 μM) for these experiments.

The Effects of a Balanced Amino Acid Mixture on Protein Synthesis as a Function of Leucine and Tryptophan Concentrations

The addition of a balanced amino acid mixture stimulated the synthesis of protein to a greater extent than did the equimolar amino acid mixture when leucine was limiting at 5 μM (Fig. 10). This indicates that the relative concentrations of amino acids are important for protein synthesis under these conditions. The results were similar regardless of whether the 10 K-S or 40 K-S, 40 K-M systems were used (Table 10, Fig. 10). The

addition of an equimolar amino acid mixture to either system resulted in no observed stimulation of protein synthesis (Table 10) beyond the control condition in which endogenous amino acids were present. It can be concluded that a balanced amino acid mixture protects the system against the imposed leucine limitation.

Cell culture experiments were also done to test whether the balanced amino acids mixture would have any effect on the synthesis of protein in an intact cell system (Fig. 11). The synthesis of protein in the early time periods by 3T3 mouse fibroblast cells in culture was significantly stimulated by replacement of the amino acids in Dulbecco's Modified Eagle's medium (DME) with a balanced mixture of amino acids. However, replacement of the amino acids in DME with an equimolar mixture of amino acids was even less effective than the normally constituted DME. These results suggest that the establishment of a physiological mixture of amino acids is important for cell-culture experiments.

A wide variety of media have been developed for cultivation of many cell types (37) and organisms (14). Essentially all of these chemically defined media contained the same ingredients; the crucial differences lie in the relative concentrations of different constituents, principally, the amino acids. No single medium has proved optimal for the growth of all cells (26,27). Holley (28) has confirmed earlier reports that limitation of several amino acids arrests 3T3 cell growth. Holley (28) postulated that at least six factors control growth by affecting availability of nutrients inside the cell, and that it is the concentrations of certain critical nutrients which actually control growth. In addition, Pardee, et al. (65), have clearly shown that deprivation of either serum or specific nutrients (glutamine, isoleucine)

can lead to a restriction of growth in "normal" cultured cells at some critical point in the G1 phase. This may be related to the fact that protein synthesis is required during the G1 phase and therefore is very sensitive to environmental and nutritional factors (66).

Recently, evidence supporting the importance of the balanced amino acid concept was provided by Seglen, et al. (67). Hepatocytes in cell culture cannot be serially passaged as can many other cell types (28). They normally remain functional for approximately 48 h before cell death occurs. However, by adding a physiologically balanced mixture of amino acids, Seglen demonstrated that rat hepatocytes remained functional and viable for greater than 72 h, a 2-fold increase in cell survival time. The observations of Seglen are similar to those obtained in our investigation with the 3T3 mouse fibroblasts (Fig. 11). These results provide further evidence of the importance of the balance of amino acids acting as a buffer in a stress situation.

The preceding studies have indicated only a quantitative effect on protein synthesis when leucine was limiting. They provide no information related to possible qualitative differences. Qualitative differences in the synthesis of protein were also observed in this investigation (Fig. 12B). The *in vitro* experiments with a 10 K-S fraction demonstrated that the addition of a balanced amino acid mixture affected synthesis primarily of the albumin fraction (Table 11). These experiments also indicated that the amount of albumin synthesized *in vitro* was less than that observed *in vivo* and also that the addition of a balanced amino acid mixture enhanced albumin synthesis when compared to the condition where an equimolar amino acid mixture was added (Table 11). Others have shown that albumin synthesis is affected by the concentration of

tryptophan (17,18). Tryptophan appears to be a regulator, controlling polysome distribution and the mRNA specific for albumin in rat liver (18).

The fact that the balanced amino acid mixture affected synthesis of albumin to a greater extent than did the equimolar mixture was interesting because tryptophan was at a much higher level in the equimolar mixture (218 μM vs 60 μM). If albumin synthesis were based only on increased tryptophan concentrations, then the synthesis should have been greater with the addition of an equimolar amino acid mixture. This was not observed. When the tryptophan concentration was varied in the presence of either a balanced or an equimolar amino acid mixture and leucine concentration was 5 μM, no stimulation of total protein synthesis was observed (Fig. 13). Small, but significant, differences in synthesis of the albumin fraction were observed (Table 13). However, the effect of the tryptophan concentration on synthesis of albumin was significantly smaller than the difference between added amino acid mixtures. Tryptophan seems to act as a secondary regulator of protein synthesis at least under the conditions of these experiments.

Experiments designed to evaluate the significance of the amino acid mixture when leucine was limiting at 5 μM have been discussed. However, leucine is normally present in the plasma and the cell at much higher concontrations. Experiments designed to determine the effect of leucine concentration demonstrated that no significant difference in the synthesis of total protein or albumin occurred as a function of either the relative concentrations of amino acids or the concentration of tryptophan, when leucine was 183 μM (Tables 12, 14). However, total protein synthesis and albumin synthesis were increased in comparison to the

condition of leucine limiting at 5 μM.

Quantitation of Albumin by Immunoprecipitative Techniques

The quantitation of albumin by gel profile analysis and immunoprecipitation methods produced different values (Table 16). A literature survey indicated that the quantity of albumin detected by different methods varied between 0.25% and 40% (50). Direct immunoprecipitation produced consistently low values for albumin (Table 16). Indirect immunoprecipitation resulted in more consistent values at higher levels than those obtained by the direct method (Table 16). However, gel analysis of the material precipitated by these methods indicated that the direct immunoprecipitation method was more specific for albumin, but accounted for less albumin than expected (Fig. 19). The indirect method precipitated more albumin and antigenically related albumin-like protein (Fig. 20). Estimation of the molecular weight of gel slices 23-28 suggests that this may be pre-proalbumin, as well as proalbumin (50). The fractions migrating before albumin are nascent peptide chains with antigenic sites that are recognized by rabbit anti-mouse albumin antisera. These methods are therefore not quantitative although each is consistent. A relative proportion of the total amount of albumin or albumin fraction synthesized can be detected. The direct method of immunoprecipitation was used in this investigation.

The Effects of Leucine Concentration on the Rate of Protein Synthesis

Where time was varied, experiments proved important to the interpretation of data related to the effect of leucine concentration and the requirement for a balanced amino acid mixture. Two time periods

seemed to be the most important when leucine was limiting at 5 μM (Fig. 21); the first 5 minute period was the most critical. The greatest difference in the dependency for amino acids was observed during this time interval. The later period (20-40 min) also was less important and after 40 minutes no significant changes in protein synthesis was observed in the presence of either a balanced amino acid mixture or an equimolar amino acid mixture. The initial incubation time established for this investigation was 40 minutes. This is characteristic of the zero order portion of the velocity curve for both conditions. The effect observed at 40 minutes, therefore, is independent of the concentration of amino acids. Rather, it is a reflection of the differences in the extent of incorporation of leucine into protein as a result of changes that occurred in the early time periods caused by the addition of an amino acid mixture.

The addition of a balanced amino acids mixture significantly stimulated synthesis of protein during early time periods when leucine was 5 μM, in comparison to the addition of an equimolar mixture of amino acids (Fig. 21). The balanced amino acid mixture partially protected the system against the limitation of low leucine concentration.

The dependency of the protein synthesizing system on the relative concentrations of other amino acids in the mixture disappeared when leucine was increased to 183 μM (Table 12). The mechanism of leucine involvement in regulation of protein synthesis is not provided by these experiments.

The rate studies when the leucine concentration was 183 μM indicate that protein synthesis was not zero order at 40 or at 80 min, but rather mixed order (Fig. 22), and that at earlier times mixed order (Fig. 27).

This suggests that leucine significantly enhanced the rate of the reaction, since first order kinetics were not observed in the 37.5 - 300 sec time period. This is an indication that a more physiological condition is present because the rate of synthesis during the early 0 - 37.5 sec period is much greater. The lag in the rate of protein synthesis at early time periods has been a source of concern with respect to the use of cell-free *in vitro* protein synthesizing systems. This is not observed during the early time period *in vivo*.

Kinetic evaluation of the concentration dependency of protein synthesis on leucine suggests that the supernatant contained two components sensitive to leucine, or a single component with multiple sites. The component with a high Km and a high Vmax is sensitive to leucine concentration over the range of its suspected physiological concentrations. The component with a low Km and a low Vmax is affected in experiments with the 40 K-S, 40 K-M system. It is possible that the high Km component, operative under fed conditions, allows for maximal stimulation of protein synthesis and is an anabolic enzyme. The low Km component could operate under conditions of leucine limitation and may be a catabolic enzyme, allowing for basal levels of protein syntehsis in liver.

The two component system might affect charging of a different leucine tRNA since there are isoaccepting species of tRNA present within the cell (4,6). The translational rate could be altered. Several investigators have observed that when leucine concentration is varied, i.e., greater than 3 μM, the charging of tRNA for glycine is inhibited (13). This indicates that an activation reaction of protein synthesis may be altered by different concentrations of other substrates; for example, the concentration of leucine effects charging of glycyl-tRNA by glycine.

The result could be an inhibition or activation of protein synthesis, changing the translational efficiency (6).

In this investigation, first order kinetics were observed for the condition where leucine was limiting (Fig. 24). If leucine was physiological, the studies indicate linear, mixed order kinetics (Figs. 27, 28).

The rate constants derived from the Michaelis evaluation and those derived from the first order treatment of the rate studies were in excellent agreement, considering the complexity of the system (Table 18). This strengthens the argument for the two component system sensitive to leucine concentration. The low Km component and the rate constant derived from it agree with the first order derived constant. The high Km value agrees with the plasma concentration of leucine obtained by amino acid analysis (Table 17).

As indicated previously, a variation of the tryptophan concentration when leucine was limiting, stimulated synthesis of protein (Fig. 13, Table 13). Qualitative and quantitative differences in synthesis were observed under these conditions, but no differences were observed with respect to polysome distribution (Table 19). This was rather surprising since tryptophan has been implicated in ribosome aggregation by several investigators using perfused tissue or intact animals (19-21). One investigator reported ribosome aggregation *in vitro* with a reticulocyte lysate system (6).

Analysis of Polysome Distribution as a Function of Leucine and Tryptophan Concentrations

Concentration changes of tryptophan did not alter synthesis of protein when leucine was physiological (Tables 12, 14). However, the

polysome distribution was affected and high molecular weight aggregates were observed (Fig. 31, Table 19). The changes in polysome size resulting from variation of tryptophan were primarily dependent on leucine concentration and secondarily dependent on tryptophan. Leucine allowed the tryptophan effect on polysome aggregation to occur. The fact that no apparent stimulation of protein synthesis was observed, even though polysome aggregation was altered, has not been resolved. However, when the tryptophan concentration was 2 μM and leucine was physiological, the polysome aggregate was much larger than aggregates observed with 2 μM tryptophan and leucine limiting. The difference in synthesis of protein between limiting conditions of leucine and physiological concentrations of leucine may be due to a permissive effect of leucine on tryptophan aggregation of polysomes. This study also indicates that the synthesis of the albumin fraction was primarily dependent on the concentration of leucine rather than tryptophan (Table 15).

Stimulation of protein synthesis, when leucine was physiological, as a function of the concentration of tryptophan is possibly a fine tuning mechanism that effects translational efficiency. Evidence suggesting that this may be true has been reported by Strair, et al. (18) and Shafritz (19). Tryptophan, in vivo, apparently controls the distribution of mRNA specific for albumin ($mRNA_{ALB}$). The $mRNA_{ALB}$ is synthesized and stored as an untranslatable messenger ribonucleoprotein fraction. The translatable $mRNA_{ALB}$ is "unmasked" and bound to the 40S-initiation complex free in the cytosol. Synthesis of albumin occurs on the endoplasmic reticulum; a membrane-polysome complex appears to be essential for albumin synthesis (18); some synthesis of albumin occurs in the absence of tryptophan (18).

Since investigations in this laboratory were primarily done with a cell-free system, the direct involvement of tryptophan in stimulation of protein synthesis could not be observed. The integrity of membrane fragments was not determined, nor was the distribution of $mRNA_{ALB}$. What can be concluded is that the tryptophan effect may not be observable except in the intact cell. Polysome aggregation could be altered, but the true tryptophan effect could not be seen.

Other studies have demonstrated that tryptophan may affect translocation of polyadenylated mRNA from the nucleus to the cytosol of hepatocytes in cell culture (20). This again provides evidence that an intact system is necessary to study this phenomena.

Further evidence regarding the necessity of an intact system to study the tryptophan effect was provided by Manchester (68). In the cell-free system, no tryptophan effect was observed. The integrity of the membranes is, therefore, critical for the evaluation of the tryptophan effect and perhaps other amino acids.

Periano, et al. (24) suggest that tryptophan may play a more general role than other amino acids in the regulation of enzyme levels in mammalian liver. This would imply that more than one regulatory mechanism involving amino acid concentrations and balance may be involved. This is exactly what is observed with leucine in our experiments.

The Effect of Increasing Supernatant Concentration on the Synthesis of Protein when the Leucine Concentration is Either 5 µM or 183 µM.

Additional evidence supportive of the concept of a regulatory role for leucine in protein synthesis was obtained from experiments in which

the supernatant concentration was changed, and the added leucine concentration remained constant. Increasing the supernatant concentration in the presence of either limiting or physiological concentrations of leucine produced different effects. Limiting conditions of leucine resulted in a signoidal velocity curve for increasing concentrations of supernatant. However, the velocity curve was hyperbolic, if the leucine concentration was physiological and the supernatant concentration was increased (Fig. 34). This supports the concept that several components of the protein synthesizing system are sensitive to leucine. It also indicates the possibility of an allosteric phenomena. Leucine may act as a positive modifier of protein synthesis since the change from low to high leucine concentrations favors a switch from a sigmoidal to a hyperbolic velocity curve (Fig. 34). It is, however, necessary to interpret these velocity curves with caution. Many investigations have been done under similar conditions, and the results have been interpreted as indicative of the physiological condition. The stressed condition imposed by the leucine limitation is extremely unphysiological. Different concentrations of leucine must be tested in the intermediate ranges between 5 μM and 183 μM to verify this hypothesis.

Effects of Adenine Nucleotides on Protein Synthesis

The ATP dependent inhibition has been observed repeatedly in this investigation independent of the amino acid composition (Table 23). Inhibition occurred at concentrations greater than the physiological concentration of 1 mM. The observed ATP inhibition has been quite controversial. Several investigators have recognized that ATP inhibits protein synthesis (69) while others have not been able to repeat this

effect (70). The protein synthesizing systems studied, however, are quite diverse with respect to tissue source, incubation time and degree of fractionation, which may explain the different experimental results. Rodemann, et al. (71) reported that two proteolytic systems exist for catabolism of amino acids in isolated skeletal muscle preparations. One system, the lysosomal system, is inhibited by specific proteases inhibitors such as leupeptin or chymostatin. The second system, the ATP dependent system, is inhibited by the transamination product of leucine, 2-ketoisocaproate. The ATP inhibition of protein synthesis is related to enhancement of the degradative reactions or protein breakdown. ATP inhibition in our investigation was observed with both leucine limiting at 5 μM and leucine at 183 μM. In Tischler's experiments concentrations of greater than 2 mM leucine were used (59).

The cyclic nucleotide, cAMP, has been implicated as both an inhibitor and an activator of protein synthesis. This nucleotide appears to exert primarily selective effects on protein synthesis in liver tissue (72). Because of our observed nucleotide inhibition by ATP and the interest in cyclic nucleotides, the effect of cAMP in the mouse liver system was studied in relation to the amino acid effects (Table 22). Cyclic AMP, when administered by itself was without effect. When theophylline, an inhibitor of the phosphodiesterase, was added, synthesis of the albumin fraction, as a percentage of total protein synthesis was enhanced (Table 21). The theophylline and cAMP response was not studied sufficiently and no interpretation can be presented. The theophylline effect, however, is interesting because as an inhibitor of phosphodiesterase it should mimic the effect of high cAMP concentrations. This did not occur, which could mean that the phosphodiesterase inhibitor is preventing

breakdown of another cyclic nucleotide such as a cGMP. The cyclic nucleotide, cGMP, has been implicated in lymphocyte activation (30) and could be involved as a regulator of protein synthesis. The guanosine nucleotide, GTP, is exclusively used for several of the reactions of protein synthesis (4).

A Low Molecular Weight Inhibitor of Protein Synthesis

In addition, a low molecular weight inhibitor has been observed in the liver cell-free system (Table 20). The nature of this inhibitor has not been determined. However, it can be partially removed by Sephadex G-25 treatment of the 10,000 x g supernatant (10 K-S). Dialysis of this fraction inhibited protein synthesis, which indicates that an essential component of the system was removed during the dialysis procedure (Table 21), and does not provide information related to the nature of the inhibitor as was anticipated. Reconstitution experiments will be necessary to characterize this inhibitor.

Recently several individuals reported that the reticulocyte lysate system contained a factor that inhibited protein synthesis if the concentration of hemin was low (6). Hemin is utilized for the synthesis of hemoglobin and is the porphyrin moiety containing iron. It is positioned in the peptide chains of hemoglobin. Several other inhibitors of protein synthesis have been observed. A double stranded RNA (dsRNA), and tetranucleotide have been shown to inhibit eukaryotic and bacterial systems respectively (4). These compounds are low molecular weight inhibitors.

Determination of Plasma Amino Acid Concentrations

The determination of the amino acid concentrations for the plasma of C3HeB/FeJ showed that the concentrations of both tryptophan and leucine vary only slightly in response to the dietary condition of the animals. Their concentration gradient is approximately one. In general, a fasting condition resulted in amino acid levels considerably higher than the fed condition. However, both aspartate, histidine and threonine concentrations decreased. The concentration of leucine was 120 μM; tryptophan was 60 μM. The leucine concentration approximates one of the Km values found for leucine in the cell-free studies (113 μM). Other investigators have demonstrated that plasma, rather than the cytosol, provides the amino acids that are directly bound to tRNA (60-64). The plasma values, then, may represent the true physiological concentrations of amino acids available for protein synthesis. This would implicate the membrane as a mediator in control of synthesis of protein. The aminoacyl-tRNA synthetases for mammalian systems have not all been purified; it could be significant to compare the Km values for the various enzymes and their amino acid substrates with the plasma concentrations of the amino acids.

Age dependent alterations were also noted for all of the amino acids (Table 27). The variation of leucine was quite small in comparison to other amino acids (108 μM - 130 μM). Greater variations between the fed and fasted conditions were also observed for all amino acids (Tables 24, 25).

In establishing a system for either cell-free protein synthesis or cell culture studies, it appears justifiable to conclude that an amino

acid mixture based on physiological values be added to the medium of interest. As with other parameters, a balanced amino acid mixture insures the investigator of one less variable to control, especially when the results must be related to the question of whether or not the experimental observations are physiologically important.

CONCLUSIONS

A cell-free system derived from mouse liver has been investigated with respect to its ability to synthesize proteins under various conditions related to amino acid supply. It can be concluded from these studies that leucine is an important regulator of protein synthesis. Furthermore, the effect of tryptophan as a regulator of polysome distribution is dependent on the concentration of leucine; it suggests a permissive role for leucine in relation to tryptophan.

Under stressful conditions, such as the limitation of leucine, the physiological balance of other amino acids becomes important. The balance of amino acids protects the protein synthesizing system, thus allowing for synthesis of protein in a pattern more indicative of the normal.

It can also be concluded that the liver cells of mice contain a low molecular weight inhibitor of protein synthesis. No further characterization of the inhibitor has been done; dialysis failed to remove the inhibitor. However, the inhibitor effect is independent of the concentration of leucine or the addition of an amino acid mixture.

Finally, these studies suggest the importance of recognizing the limitations of the protein synthesizing systems currently used, if a

physiological correlation is desired. The limitation of amino acids imposes an abnormal condition on the system; the system response is stressed and most probably unphysiological. The conclusions obtained under these conditions must be carefully interpreted. Model systems must be validated for the physiological significance of results obtained with them.

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APPENDIX A

Algebraic System Program for the Processing of Amino Acid Data

The program described was written for a Texas Instruments TI 59 Calculator attached to a PC-100 Printing Unit. It enabled the user to calculate the amino acid concentration in plasma or urine in mg/100 ml or mM units. The only data entered were peak heights and widths at half peak heights; all data was then processed automatically. Correction factors from calibration runs were processed separately and stored on magnetic tape for entry into the program. Since the calculator contains only 59 memory registers, data input for correction factors and amino acid constants occurred in different portions of the main program which requires 150 steps and 59 memory registers. The sequence for data entry and the memory address for specific amino acid data is shown in Table A-I. A schematic diagram of the program is presented in Table A-II. User instructions are given in Table A-III. The program listing for the calculation of amino acid concentrations is shown in Table A-IV. Labels and data cards are given in Table A-V.

Table A-1

Memory Addresses of Amino Acid Data[1,2]

Amino Acid	Total Height (H)	Width ½H(W)	Correction Factor	Amino Acid Constants[3]
ASPARTIC	01	21	41	41
THREONINE	02	22	42	42
SERINE	03	23	43	43
GLUTAMIC	04	24	44	44
GLYCINE	05	25	45	45
ALANINE	06	26	46	46
VALINE	07	27	47	47
CYSTEINE	08	28	48	48
METHIONINE	09	29	49	49
ISOLEUCINE	10	30	50	50
LEUCINE	11	31	51	51
TYROSINE	12	32	52	52
PHENYLALANINE	13	33	53	53
LYSINE	14	34	54	54
HISTIDINE	15	35	55	55
TRYPTOPHAN	16	36	56	56
ARGININE	17	37	57	57
GLUTAMINE	18	38	58	58

[1] Registers 19 and 59 are reserved for the dilution factor and sample volume injected.

[2] Registers 01 - 18 are used continuously throughout the program for storage of calculated results. Therefore, when H x W is calculated, the product is stored in registers 01 - 18; the total heights initially stored in these locations are no longer necessary.

[3] Registers 41 - 58 are used twice at different locations in the program. Therefore, data input to these registers is done separately when either correction factors or amino acid constants are needed.

Table A-II

Schematic Diagram of Amino Acid Program

Program Steps	Entry	Description
000 - 112	Directions for Alpha Print Code	Label for Amino Acid Chromatogram.
Reset		
000 - 040	H x W value for Norleucine Standard, nanomoles of norleucine added.	Correction factor for norleucine based on regression analysis from standard curve.
		Counter set for indexing of registers.
041 - 076	Data Entry: H and W and ½H for each amino acid	The H x W factor for each amino acid is calculated and corrected, based on the correction factor for norleucine.
076 - 116	Data Entry: Correction factors for each amino acid are entered in registers 41 - 58. Dilution factor is entered in register 19 and sample volume in register 59	Nanomoles of each amino acid are calculated, and mM value is printed.
116 - 150	Data Entry: Constants for conversion of mM values to mg/100 ml	Counter Reset Calculation of mg/100ml plasma values, and values printed.

Table A-III

Register Contents and Labels for Amino Acid Analysis

Register	Contents	Labels	Contents
R19	Dilution factor	A'	C_{HW} - Norleucine
R39	Correction factor for norleucine	B'	Nanomole of norleucine injected.
R59	Sample volume injected	C'	Counter set. Height and width of each amino acid entered in R01 - 18 and R21 - 38 respectively.
		A	Height x width calculated for each amino acid and stored in R01 - 18.
		B	Counter reset. Correction factors for each amino acid input.
		C	Nanomoles and mM value of each amino acid calculated.
		D	Counter reset. Amino acid constants entered.
		E	Plasma value in mg/100 ml printed.

Table A-IV

Program for Amino Acid Analysis

Line	Key	Entry	Line	Key	Entry	Line	Key	Entry	Line	Key	Entry
000	76	LBL	038	42	STD	076	91	R/S	114	67	EQ
001	16	A'	039	20	20	077	76	LBL	115	13	C
002	42	ST□	040	91	R/S	078	12	B	116	91	R/S
003	39	39	041	76	LBL	079	00	0	117	76	LBL
004	91	R/S	042	11	A	080	42	ST□	118	14	D
005	76	LBL	043	01	1	081	00	00	119	00	0
006	17	B'	044	08	8	082	04	4	120	42	ST□
007	65	X	045	32	XIT	083	00	0	121	00	00
008	09	9	046	01	1	084	42	ST□	122	04	4
009	93	.	047	44	SUM	085	40	40	123	00	0
010	07	7	048	00	00	086	91	R/S	124	42	ST□
011	06	6	049	44	SUM	087	76	LBL	125	40	40
012	85	+	050	20	20	088	13	C	126	91	R/S
013	01	1	051	73	RC*	089	01	1	127	76	LBL
014	06	6	052	00	00	090	44	SUM	128	15	E
015	05	5	053	65	X	091	00	00	129	01	1
016	93	.	054	73	RC*	092	44	SUM	130	44	SUM
017	06	6	055	20	20	093	40	40	131	00	00
018	05	5	056	95	=	094	73	RC*	132	44	SUM
019	95	=	057	55	÷	095	00	00	133	40	40
020	42	ST□	058	43	RCL	096	55	÷	134	73	RC*
021	41	41	059	39	39	097	73	RC*	135	00	00
022	91	R/S	060	95	=	098	40	40	136	65	X
023	76	LBL	061	55	÷	099	95	=	137	73	RC*
024	18	C'	062	43	RCL	100	55	÷	138	40	40
025	43	RCL	063	42	42	101	43	RCL	139	95	=
026	39	39	064	95	=	102	59	59	140	99	PRT
027	55	÷	065	99	PRT	103	95	=	141	72	ST*
028	43	RCL	066	72	ST*	104	65	X	142	00	00
029	41	41	067	00	00	105	43	RCL	143	66	PAU
030	95	=	068	66	PAU	106	19	19	144	66	PAU
031	42	ST□	069	66	PAU	107	95	=	145	43	RCL
032	42	42	070	43	RCL	108	99	PRT	146	00	00
033	00	0	071	00	00	109	72	ST*	147	22	INV
034	42	ST□	072	22	INV	110	00	00	148	67	EQ
035	00	00	073	67	EQ	111	43	RCL	149	15	E
036	02	2	074	11	A	112	00	00	150	91	R/S
037	00	0	075	98	ADV	113	22	INV			

Table A-V

Data Card for Alpha Print

Line	Key	Entry	Line	Key	Entry	Line	Key	Entry
000	76	LBL	037	07	7	074	02	2
001	11	A	038	69	▯P	075	02	2
002	69	▯P	039	03	03	076	02	2
003	00	00	040	04	4	077	03	3
004	01	1	041	05	5	078	05	5
005	03	3	042	03	3	079	69	P
006	03	3	043	06	6	080	02	02
007	00	0	044	02	2	081	01	1
008	02	2	045	04	4	082	03	3
009	04	4	046	03	3	083	03	3
010	03	3	047	06	6	084	00	0
011	01	1	048	00	0	085	00	0
012	03	3	049	00	0	086	00	0
013	02	2	050	00	0	087	00	0
014	69	▯P	051	00	0	088	00	0
015	01	01	052	69	▯P	089	03	3
016	00	0	053	04	04	090	01	1
017	00	0	054	69	▯P	091	03	3
018	01	1	055	05	05	092	02	2
019	03	3	056	98	ADV	093	04	4
020	01	1	057	01	1	094	00	0
021	05	5	058	05	5	095	69	▯P
022	02	2	029	02	2	096	03	03
023	04	4	060	03	3	097	03	3
024	01	1	061	03	3	098	02	2
025	06	6	062	05	5	099	04	4
026	69	▯P	063	03	3	100	00	0
027	02	02	064	02	2	101	00	0
028	00	0	065	03	3	102	00	0
029	00	0	066	00	0	103	00	0
030	01	1	067	69	P	104	00	0
031	03	3	068	01	01	105	00	0
032	03	3	069	01	1	106	00	0
033	01	1	070	03	3	108	04	4
034	01	1	071	03	3	109	69	▯P
035	03	3	072	07	7	110	05	05
036	02	2	073	03	3	111	98	ADV
						112	91	R/S

APPENDIX B

Fortran Program for the Processing of Single or Double Isotopes Data from Gel Electrophoresis

A program has been developed for the purpose of processing data obtained from a liquid scintillation counter. The program is written in Fortran for use with the Burroughs 6700 computer. It processes data obtained from electrophoretic gel experiments in which a single isotope or two isotopes are present. The following functions are performed by the program:

a) Counts are background-corrected, and the degree of crossover is adjusted (if the experiment is double isotope) on the basis of linear regression analysis;

b) The total isotope on each gel slice was determined whether single or double isotope was used, and the percent value for each gel slice was calculated;

c) If the gel experiment was double isotope, the ratio of each isotope in the gel slice was obtained; (This facilitated the comparison of experiments regardless of the level of individual isotope incorporation.)

d) Gel results were graphed;

e) Area integration was done using Simpson's approximation for area, and area integration was converted into percent;

f) If the values obtained from the liquid scintillation counter were small, and the counting error was greater than 10%, the results from the gel slice could be added to the following gel, and the approximate counting error could then be calculated. (This was the purpose

of the subroutine COCALC, i.e., collapsed calculations.)

All results were printed on the line printer.

The program is presented in Table B-I.

Input was accomplished with the card reader; only the count level, external standard ratio and counting error were necessary. However, Input/Output could be calculated from Hazeltine-Textronix units if file statements were changed and different graph subroutines were used.

The program was stored on disc.

Fortran Program for the Processing of Single or Double Isotopes Data from Gel Electrophoresis

```
B6700/B7700  F O R T R A N  C O M P I L A T I O N  M A R K  2.7.480   MONDAY, 03/01/76   03:10 PM

FILE  5=JPMCG,UNIT=READER                                              C  00000001
                                                                         FIB IS 0007 LONG
FILE  6=800,UNIT=PRINTER                                               C  00000002
C DIMENSION STATEMENT AND INPUT                                        C  00000003
                                                                         FIB IS 0006 LONG
                                                                       START OF SEGMENT 002
                                                                     FORMAT SEGMENT IS 00F5 LONG
      DIMENSION  A(75),B(75),C(75),D(75),E(75),F(75),G(75),M(75),Q(75) C  0021000010
      DIMENSION R(75),U(75),S(75),T(75),Y(5,100),V(75),W(75),X(75)     C  0021000010
      DIMENSION O(300),PCT(200),ST1(100),ST2(100)                      C  0021000010
      COMMON  NGEL,B,E,G,U,S,T,M,V,X,Y,L,NGELBL,O                      C  0021000010
      DATA M3BKG/16.00/,C14BKG/10.00/                                  C  0021000010
      EQUIVALENCE (NGR,M),(NF,L)                                       C  0021000010
C  INDEX AND INITIALIZE COUNTER TO SLICE ONE FOR INPUT .                C  0021000010
      N=2                                                              C  0021000010
      NGEL=1                                                           C  0021000015
      N=35                                                             C  0021000119
      NGELBL=4                                                         C  0021000214
      L=N/2                                                            C  0021000411
C  DETERMINE IF EXPERIMENT INVOLVES DOUBLE OR SINGLE LABEL.             C  0021000612
      IF(NGELBL)2,3,4                                                  C  0021000612
C  C14 COUNTS ONLY AND EXTERNAL STANDARD.                               C  0021000A11
    2 DO 6 I=NGEL,L                                                    C  0021000A11
      READ(5,7)D(I),R(I),Q(I)                                          C  0021000C10
    7 FORMAT(3F10.2)                                                   C  0021001A12
    6 CONTINUE                                                         C  0021001A12
      GO TO 23                                                         C  0021001D10
C  H3 COUNTS ONLY AND EXTERNAL STANDARD.                                C  0021001D13
    3 DO 8 I=NGEL,L                                                    C  0021001D13
      READ(5,9)A(I),R(I),Q(I)                                          C  0021001F10
    9 FORMAT(3F10.2)                                                   C  0021002D12
    8 CONTINUE                                                         C  0021002D12
      GO TO 13                                                         C  0021003010
C  H3 AND C14 COUNTS AND EXTERNAL STANDARD                              C  0021003013
    4 DO 10 I=NGEL,L                                                   C  0021003013
      READ(5,11)A(I),D(I),R(I),Q(I)                                    C  0021003210
   11 FORMAT(4F10.2)                                                   C  0021004312
   10 CONTINUE                                                         C  0021004312
C  CORRECT COUNTS FOR BACKGROUND AND IF DOUBLE LABEL FOR THE AMOUNT OF  C  0021004610
C    CROSSOVER.                                                        C  0021004610
C CROSSOVER CALCULATIONS BASED ON REGRESSION ANALYSIS                  C  0021004610
      DO 12 I=NGEL,L                                                   C  0021004610
      Y1=3.805*R(I)+35.815                                             C  0021004710
      E(I)=(D(I)*100.00)/Y1                                            C  0021004C14
      Y2=2.425*R(I)+30.357                                             C  0021005011
      XOVER=(E(I)*Y2)/100.00                                           C  0021005514
      Y3=1.877*R(I)+33.745                                             C  0021005812
      B(I)=(A(I)*100.00)/Y3                                            C  0021005D14
      B(I)=B(I)-(XOVER*M3BKG)                                          C  0021006014
   12  CONTINUE                                                        C  0021006510
      GO TO 38                                                         C  0021006514
C  CORRECT COUNTS FOR EXPERIMENTS USING H3 ONLY.                        C  0021006611
   13 DO 14 I=NGEL,L                                                   C  0021006611
      Y1=1.877*R(I)+33.745                                             C  0021006810
      B(I)=(A(I)*100.00)/Y1                                            C  0021006D14
      B(I)=B(I)-M3BKG                                                  C  0021007014
   14 CONTINUE                                                         C  0021007213
      GO TO 24                                                         C  0021007511
C  CORRECT COUNTS FOR EXPERIMENTS USING C14 ONLY.                       C  0021007514
   23 DO 15 I=NGEL,L                                                   C  0021007514
      Y1=3.805*R(I)+33.815                                             C  0021007710
      E(I)=(D(I)*100.00)/Y1                                            C  0021007C14
```

```
      E(I)=E(I)-C14BKG                                                  C  0021008011
   15 CONTINUE                                                          C  0021008215
      GO TO 58                                                          C  0021008511
C  DETERMINE TOTAL C14 AND H3 COUNTS ON ACRYLAMIDE GEL ,WHETHER         C  0021008514
C   SINGLE OR DOUBLE LABELLING.                                         C  0021008519
   58 IF(NGELBL)25,24,25                                                C  0021008514
   25 C14T=0.0                                                          C  0021008712
      DO 16 I=NGEL,L                                                    C  0021008810
      C14T=C14T+E(I)                                                    C  0021008910
   16 CONTINUE                                                          C  0021008B11
      IF(NGELBL)30,24,24                                                C  0021008D15
   24 H3T=0.0                                                           C  0021008F13
      DO 21 I=NGEL,L                                                    C  0021009011
      H3T=H3T+B(I)                                                      C  0021009210
   21 CONTINUE                                                          C  0021009414
      GO TO 28                                                          C  0021009612
C OBTAIN PERCENT OF H3 AND C14 IN GEL SLICES---PERCENT CALCULATIONS     C  0021009615
C   FACILITATES COMPARISON BETWEEN DIFFERENT EXPERIMENTS.               C  0021009615
   28 DO 17 I=NGEL,L                                                    C  0021009815
      C(I)=(B(I)/H3T)*100.00                                            C  0021009810
   17 CONTINUE                                                          C  0021009B10
      IF(NGELBL)30,42,30                                                C  0021009D14
   30 DO 29 I=NGEL,L                                                    C  0021009F12
      F(I)=(E(I)/C14T)*100.00                                           C  002100A110
   29 CONTINUE                                                          C  002100A413
      IF(NGELBL)42,42,41                                                C  002100A711
C  CALCULATION OF H3/C14 RATIOS---FACILITATES COMPARISON OF EXPERIMENTS C  002100A912
C   REGARDLESS OF LEVEL OF INDIVIDUAL ISOTOPE INCORPORATION AND         C  002100A912
C   IDENTIFIES DEVIATIONS FROM IDENTITY OF TWO ISOTOPE PROFILES .THIS   C  002100A912
C   IS A NORMALIZED RATIO.                                              C  002100A912
C   CALCULATION OF RATIO FOR TOTAL GEL.                                 C  002100A912
   41 RATGEL=H3T/C14T                                                   C  002100A912
C  CALCULATION OF RAW RATIO FOR EACH SLICE.                            C  002100AA14
      DO 27 I=NGEL,L                                                    C  002100AA14
      G(I)=B(I)/E(I)                                                    C  002100AC10
   27 CONTINUE                                                          C  002100AF12
C   CALCULATION OF NORMALIZED RATIO FOR EACH GEL.                       C  0021008210
      DO 18 I=NGEL,L                                                    C  0021008210
      H(I)=G(I)/RATGEL                                                  C  0021008310
   18 CONTINUE                                                          C  0021008515
   42 IF(NGELBL)501,502,505                                             C  0021008813
  505 WRITE(6,120)H3T,C14T,RATGEL                                       C  0021008B15
  120 FORMAT(1X,'EXPERIMENT--GCF-UOP/IV,022776---C3HEB/FEJ---BAL/EQUI-'  C  002100C512
     1'IN VITRO--AMINO ACID BALANCE.'/1X,T11,'TOTAL H3 ON GEL='F10.2     C  002100C512
     2,T50,'TOTAL C14 ON GEL='F9.2,T80,'H3/C14='F7.2,/1X,'NO',T9,'H3',  C  002100C512
     3 T16,'H3=COR',T26,'PCT H3',T36,'C14',T45,'C14=COR',T55,'PCT=C14',  C  002100C512
     4 T70,'H3/C14',T84,'ERROR=PCT',T96,'EXTERNAL',/1X,T66,'RAW',T74,   C  002100C512
     5 'NORMAL',T96,'STD,')                                             C  002100C512
      DO 150 I=NGEL,L                                                   C  002100C912
      WRITE(6,210)I,A(I),B(I),C(I),D(I),E(I),F(I),G(I),H(I),Q(I),R(I)   C  002100C710
  210 FORMAT(1X,I2,1X,F8.2,2X,F8.2,2X,F5.2,5X,F7.2,4X,F7.2,4X,F5.2,     C  002100E312
     14X, F5.2,5X,F5.2,6X,F5.2,7X,F5.2)                                 C  002100E312
  150 CONTINUE                                                          C  002100E512
      GO TO 510                                                         C  002100E610
  501 WRITE(6,503)C14T                                                  C  002100E613
  503 FORMAT(1X,'EXPERIMENT-    -GCF-UOP/IV, DATE  .--C3HEB/FEJ--'      C  002100ED12
     1'CONTROL--IN VITRO---PROTEIN BIOSYNTHESIS---GEL NO  ',/1X,T40,    C  002100ED12
     2'TOTAL C14 ON GEL=',F8.2,/1X,T33,'NO',T42,'C-14',T51,'C14=COR'     C  002100ED12
     3,T62,'PCT',T69,'ERROR=PCT',T81,'EXTERNAL',/1X,T84,'STD,')         C  002100ED12
      DO 507 I=NGEL,L                                                   C  002100ED12
      WRITE(6,506) I,D(I),E(I),F(I),G(I),R(I)                           C  002100EF10
  506 FORMAT(1X,T33,I2,4X,F8.2,3X,F8.2,3X,F5.2,5X,F5.2,8X,F5.2)         C  0021010112
  507 CONTINUE                                                          C  0021010112
      GO TO 510                                                         C  0021010410
  502 WRITE(6,508)H3T                                                   C  0021010413
  508 FORMAT(1X,'EXPERIMENT-    -GCF-UOP/IV, DATE  .--C3HEB/FEJ---'     C  0021010812
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  203 Y(I,J)=INT(Y(I,J)*FA)                                          C  0021020D10
  202 CONTINUE                                                       C  0021020E12
C  WRITE HEADINGS FOR GRAPH.                                        C  0021020E13
      IF(NGELBL)521,522,520                                          C  0021020E13
  521 WRITE(6,525)                                                   C  0021020E15
  525 FORMAT(1H1,1X,T48,'C14 DPM=A',/,)                              C  0021021012
      GO TO 213                                                      C  0021021012
  522 WRITE(6,526)                                                   C  0021021015
  526 FORMAT(1H1,1X,T48,'H3  DPM=A',/,)                              C  0021021512
      GO TO 213                                                      C  0021021512
  520 WRITE(6,300)                                                   C  0021021515
  300 FORMAT(1H1,1X,T45,'C14 DPM=A,H3 DPM=B',/,)                     C  0021021A12
  213 CONTINUE                                                       C  0021021A12
      CALL PLOT (Y,M,NF,NS)                                          C  0021021A12
      CALL AREA(Y,L,O,NGFL/NGELBL,PCT)                               C  0021021E10
C   GRAPH OF H3/C14==USE NORMALIZED RATIO.                           C  0021022410
      IF(NGELBL)158,158,170                                          C  0021022410
  170 DO 131 I=NGEL,1                                                C  0021022514
      DO 230 J=NGEL,L                                                C  0021022710
  230 Y(I,J)=M(J)                                                    C  0021022810
  131 CONTINUE                                                       C  0021022E12
C  DETERMINE MAX FOR H3/C14 RATIO.                                  C  0021023012
      DO 216 J=1,NA                                                  C  0021023012
      K=J                                                            C  0021023110
      KA=J+1                                                         C  0021023115
      DO 219 I=KA,L                                                  C  0021023311
  219 IF(M(I),LT,M(K))  K=I                                          C  0021023610
      TEMP=M(J)                                                      C  0021023A11
      M(J)=M(K)                                                      C  0021023B14
  216 M(K)=TEMP                                                      C  0021023E10
      RATMAX=M(L)                                                    C  0021024115
      NB=L                                                           C  0021024119
      FA=99.00/RATMAX                                                C  0021024511
      NS=INT(RATMAX*FA)                                              C  0021024613
      M=1                                                            C  0021024810
      DO 305 I=NGEL,1                                                C  0021024814
      DO 306 J=NGEL,L                                                C  0021024A10
  306 Y(I,J)=INT(Y(I,J)*FA)                                          C  0021024B10
  305 CONTINUE                                                       C  0021025112
      WRITE(6,301)                                                   C  0021025312
  301 FORMAT(1H1,1X,T48,'H3/C14,A=',A,',',/,)                        C  0021025712
      CALL PLOT (Y,M,NF,NS)                                          C  0021025712
C  SCALE FACTORS FOR INDIVIDUAL GRAPHS AFTER TRANSFER TO Y ARRAY.   C  0021025810
      IF(NGELBL)158,158,430                                          C  0021025810
  430 DO 431 I=1,1                                                   C  0021025C14
      DO 432 J=NGEL,L                                                C  0021025E10
  432 Y(I,J)=BT1(J)                                                  C  0021025F10
  431 CONTINUE                                                       C  0021026512
      M=1                                                            C  0021026712
      NB=L                                                           C  0021026810
      FA=100.00/C14MAX                                               C  0021026912
      NS=INT(C14MAX*FA)                                              C  0021026A14
      DO 433 I=1,1                                                   C  0021026C11
      DO 434 J=NGEL,L                                                C  0021026D10
  434 Y(I,J)=INT(Y(I,J)*FA)                                          C  0021026E10
  433 CONTINUE                                                       C  0021027412
      WRITE(6,435)                                                   C  0021027612
  435 FORMAT(1H1,1X,T45,'C14 DPM=A',/,)                              C  0021027A12
      CALL PLOT(Y,M,NF,NS)                                           C  0021027A12
      DO 436 I=1,1                                                   C  0021027E10
      DO 437 J=NGEL,L                                                C  0021027F10
  437 Y(I,J)=BT2(J)                                                  C  0021028010
  436 CONTINUE                                                       C  0021028612
      FA=100.00/H3MAX                                                C  0021028812
      NS=INT(H3MAX*FA)                                               C  0021028914
      DO 438 I=1,1                                                   C  0021028B11
      DO 439 J=NGEL,L                                                C  0021028C10
  439 Y(I,J)=INT(Y(I,J)*FA )                                         C  0021028D10
  438 CONTINUE                                                       C  0021029312
      WRITE(6,440)                                                   C  0021029512
  440 FORMAT(1H1,1X,T45,'H3 DPM=A',/,)                               C  0021029912
      CALL PLOT(Y,M,NF,NS)                                           C  0021029912
  158 STOP                                                           C  0021029D10
      END                                                            C  0021029D15
                                                                  SEGMENT 002 IS 02CD LONG
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C  SUBROUTINE AREA                                                    C  002:02CD:0
                                                                       START OF SEGMENT 005
      SUBROUTINE AREA(Y,L,O,NGEL,NGELBL,PCT)                           C  005:0000:0
      DIMENSION Y(5,1n0),O(300) ,PCT(200)                              C  005:0000:0
      X=0.0                                                            C  005:0000:0
      SUMI=0.0                                                         C  005:0000:0
      DX=1.00                                                          C  005:0001:2
C  DO LOOP FOR CALCULATIONS OF INTEGRAL                                C  005:0002:0
      DO 10 I=NGEL,1                                                   C  005:0002:0
      DO 11 J=NGEL,L                                                   C  005:0003:0
      FX=Y(I,J)                                                        C  005:0006:0
      IF(J.EQ.NGEL.OR.J.EQ.L ) GO TO 8                                 C  005:0007:1
      M=J/2                                                            C  005:0009:1
      K=2*M                                                            C  005:000B:0
      IF(K.EQ.J)GO TO 7                                                C  005:000C:3
      W=(2./3.)*DX                                                     C  005:000D:3
      GO TO 9                                                          C  005:000F:2
    7 W=(4./3.)*DX                                                     C  005:000F:5
      GO TO 9                                                          C  005:0011:4
    8 W=(1./3.)*DX                                                     C  005:0012:1
    9 WHT=(FX*W)                                                       C  005:0013:5
      SUMI=SUMI+(FX*W)                                                 C  005:0015:1
      O(J)=WHT                                                         C  005:0017:0
      X=X+DX                                                           C  005:0019:1
   11 CONTINUE                                                         C  005:001B:3
   10 CONTINUE                                                         C  005:001C:4
C  CONVERT VALUES FROM AREA INTEGRATION INTO PERCENT.                  C  005:001E:4
      WRITE(6,32)SUMI                                                  C  005:001E:4
   32 FORMAT(1H1,1X,T45,'GRAPH ANALYSIS',/1X,T41,'DETERMINATION OF'    C  005:0025:2
     1'AREA',/1X,T32,'TOTAL AREA OF A =',F10.2,/1X,T36,'NO',T46,'AREA',C  005:0025:2
     2 T58,'PERCENT',)                                                 C  005:0025:2
      DO 31 I=NGEL,L                                                   C  005:0025:2
      IF(O(I),LE.0.00) GO TO 91                                        C  005:0027:0
      PCT(I)=(O(I)/SUMI)*100.00                                        C  005:0029:2
      GO TO 93                                                         C  005:002D:0
   91 PCT(I)=0.00                                                      C  005:002E:1
   93 WRITE(6,55)I,O(I),PCT(I)                                         C  005:0033:1
   55 FORMAT(1X,T35,I3,2X,F10.2,8X,F5.2)                               C  005:003C:2
   31 CONTINUE                                                         C  005:003C:2
      IF(NGELBL)30,30,25                                               C  005:003F:3
   25 X=0.0                                                            C  005:003F:0
      DX=1.00                                                          C  005:0040:2
      SUMI=0.0                                                         C  005:0041:0
      DO 12 I=2,2                                                      C  005:0041:4
      DO 13 J=NGEL,L                                                   C  005:0045:0
      FX=Y(I,J)                                                        C  005:0048:0
      IF(J.EQ.NGEL.OR.J.EQ.L ) GO TO 80                                C  005:0047:1
      M=J/2                                                            C  005:0049:3
      K=2*M                                                            C  005:004B:0
      IF(K.EQ.J) GO TO 70                                              C  005:004C:3
      W=(2./3.)*DX                                                     C  005:004D:3
      GO TO 90                                                         C  005:004F:2
   70 W=(4./3.)*DX                                                     C  005:004F:5
      GO TO 90                                                         C  005:0051:0
   80 W=(1./3.)*DX                                                     C  005:0052:1
   90 WHT=(FX*W)                                                       C  005:0055:5
      SUMI=SUMI+(FX*W)                                                 C  005:0055:1
      O(J)=WHT                                                         C  005:0057:0
      X=X+DX                                                           C  005:0059:1
   13 CONTINUE                                                         C  005:005B:3
   12 CONTINUE                                                         C  005:005C:4
      WRITE(6,65)SUMI                                                  C  005:005E:5
   65 FORMAT(1H1,1X,T45,'GRAPH ANALYSIS',/1X,T41,'DETERMINATION OF'    C  005:0065:2
     1'AREA',/1X,T32,'TOTAL AREA OF B =',F10.2,/1X,T36,'NO',T46,'AREA',C  005:0065:2
     2 T58,'PERCENT',)                                                 C  005:0065:2
      DO 41 I=NGEL,L                                                   C  005:0065:2
      IF(O(I),LE.0.00) GO TO 92                                        C  005:0067:0
      PCT(I)=(O(I)/SUMI)*100.00                                        C  005:0069:2
      GO TO 94                                                         C  005:006D:4
   92 PCT(I)=0.00                                                      C  005:006E:1
   94 WRITE(6,56)I,O(I),PCT(I)                                         C  005:0070:1
   56 FORMAT(1X,T35,I3,2X,F10.2,8X,F5.2)                               C  005:007C:2
   41 CONTINUE                                                         C  005:007C:2
   30 RETURN                                                           C  005:007E:5
      END                                                              C  005:007F:0
                                                                      SEGMENT 005 IS 008F LONG
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C  SUBROUTINE COLLAPSED CALCULATIONS,                                  C  005:008F:0
                                                                        START OF SEGMENT 006
      SUBROUTINE COCALC                                                 C  006:0000:0
      DIMENSION B(75),F(75),Q(75),U(75),S(75),T(75),W(75),V(75),X(75)   C  006:0000:0
      COMMON NGEL,B,F,Q,U,S,T,W,V,X,L,NGELRL                            C  006:0000:0
      IF(NGEL)91,92,93                                                  C  006:0000:0
   93 DO 19 I=NGEL,L                                                    C  006:0003:0
      IF(Q(I),LT,5,00)GO TO 26                                          C  006:0004:0
      YX=B(I)                                                           C  006:0006:1
      VX=Q(I)                                                           C  006:0007:2
      ZX=F(I)                                                           C  006:0009:0
      M=I+1                                                             C  006:000A:4
      DO 20 I=NGEL,L                                                    C  006:000C:0
      U(M)=(VX+Q(M))/2,00                                               C  006:000D:0
      W(M)=YX+B(M)                                                      C  006:0010:4
      X(M)=ZX+F(M)                                                      C  006:0013:5
      IF(U(M),LT,5,00)GO TO 25                                          C  006:0016:5
      VX=U(M)                                                           C  006:0019:0
      YX=W(M)                                                           C  006:001A:4
      ZX=X(M)                                                           C  006:001C:5
      GO TO 20                                                          C  006:001E:2
   25 V(M)=U(M)                                                         C  006:001F:5
      S(M)=W(M)                                                         C  006:0021:4
      T(M)=X(M)                                                         C  006:0024:4
      I=M+1                                                             C  006:0027:4
      GO TO 19                                                          C  006:0029:0
   20 CONTINUE                                                          C  006:0029:3
   26 V(I)=Q(I)                                                         C  006:002C:1
      S(I)=B(I)                                                         C  006:002F:0
      T(I)=F(I)                                                         C  006:0031:2
   19 CONTINUE                                                          C  006:0034:1
      GO TO 100                                                         C  006:0036:5
C  C14 ONLY,                                                            C  006:0037:2
   91 DO 95 I=NGEL,L                                                    C  006:0037:2
      IF(Q(I),LT,5,00)GO TO 96                                          C  006:0039:0
      VX=Q(I)                                                           C  006:003B:1
      ZX=F(I)                                                           C  006:003C:5
      M=I+1                                                             C  006:003E:3
      DO 97 I=NGEL,L                                                    C  006:003F:5
      U(M)=(VX+Q(M))/2,00                                               C  006:0041:0
      X(M)=ZX+F(M)                                                      C  006:0044:0
      IF(U(M),LT,5,00)GO TO 101                                         C  006:0046:0
      VX=U(M)                                                           C  006:0049:1
      ZX=X(M)                                                           C  006:004B:5
      GO TO 97                                                          C  006:004D:4
  101 V(M)=U(M)                                                         C  006:004F:1
      T(M)=X(M)                                                         C  006:0051:0
      I=M+1                                                             C  006:0054:0
      GO TO 95                                                          C  006:0055:2
   97 CONTINUE                                                          C  006:0055:5
   96 V(I)=Q(I)                                                         C  006:0058:1
      T(I)=F(I)                                                         C  006:0058:2
   95 CONTINUE                                                          C  006:005E:1
      GO TO 100                                                         C  006:0060:5
C  H3 ONLY==,                                                           C  006:0061:2
   92 DO 98 I=NGEL,L                                                    C  006:0061:2
      IF(Q(I),LT,5,00)GO TO 102                                         C  006:0063:0
      VX=Q(I)                                                           C  006:0065:1
      YX=B(I)                                                           C  006:0066:5
      M=I+1                                                             C  006:0068:0
      DO 103 I=NGEL,L                                                   C  006:0069:2
      U(M)=(VX+Q(M))/2,00                                               C  006:006B:0
      W(M)=YX+B(M)                                                      C  006:006F:4
      IF(U(M),LT,5,00)GO TO 104                                         C  006:0071:3
      VX=U(M)                                                           C  006:0073:4
      YX=W(M)                                                           C  006:0075:2
      GO TO 103                                                         C  006:0077:1
  104 V(M)=U(M)                                                         C  006:0077:4
      S(M)=W(M)                                                         C  006:007A:3
      I=M+1                                                             C  006:007D:3
      GO TO 98                                                          C  006:007E:5
  103 CONTINUE                                                          C  006:007F:2
  102 V(I)=Q(I)                                                         C  006:0082:0
      S(I)=B(I)                                                         C  006:0084:5
   98 CONTINUE                                                          C  006:0087:1
  100 RETURN                                                            C  006:0089:5
      END                                                               C  006:008A:2
                                                                      SEGMENT 006 IS 0090 LONG
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C  SUBROUTINE FOR PLOTTING.                                          C  006:009010
                                                                     START OF SEGMENT 007
      SUBROUTINE PLOT(Y,M,NF,NS)                                     C  007:000010
      DIMENSION Y(5,100),LINE(101),L(11),JL(5)                       C  007:000020
      DATA (JL(I),I=1,5)/1HA,1HB,1HC,1HD,1HE/,JN,JP,JI,JBLANK,JZ/    C  007:000030
     11H=,1H+,1HI,1H ,1HS/                                           C  007:000040
      DO 99 I=1,101                                                  C  007:000050
      LINE(I)=JBLANK                                                 C  007:000110
   99 CONTINUE                                                       C  007:000210
      N=0                                                            C  007:000415
C    PRINT ORDINATE SCALE.                                           C  007:000515
      DO 101 I=1,11                                                  C  007:000515
      L(I)=10*I-110+NS                                               C  007:000710
  101 CONTINUE                                                       C  007:000A12
      WRITE(6,105)(L(I),I=1,11)                                      C  007:000C15
  105 FORMAT(3X,11(I8,6X),6HY(1,I))                                  C  007:001012
      GO TO 115                                                      C  007:001812
  110 IF(N/10=(N+1)/10)125,125,115                                   C  007:001815
C    CONSTRUCT  ORDINATE GRAPH LINE.                                 C  007:001C10
  115 ND=0                                                           C  007:001C10
      DO 120 I=1,10                                                  C  007:001C14
      ND=ND+1                                                        C  007:001E10
      LINE(ND)=JP                                                    C  007:001F12
      DO 120 J=1,9                                                   C  007:002014
      ND=ND+1                                                        C  007:002210
  120 LINE(ND)=JN                                                    C  007:002312
      LINE(101)=JP                                                   C  007:002912
      IF(N)135,121,135                                               C  007:002A14
  121 WRITE(6,170)N,LINE                                             C  007:002B15
      GO TO 185                                                      C  007:003412
C  CONSTRUCT ONE LINE OF ABSCISSA GRAPH LINE$.                       C  007:003415
  125 DO 130 I=1,101,10                                              C  007:003415
      LINE(I)=JI                                                     C  007:003610
  130 CONTINUE                                                       C  007:003714
C  CHANGE NUMERICAL DATA TO LETTERS.                                 C  007:003A10
  135 DO 160 I=1,M                                                   C  007:003A10
      XNS=NS                                                         C  007:003B10
      JA=Y(I,N)+101.49999-XNS                                        C  007:003B15
      IF(JA+101)140,155,145                                          C  007:004112
  140 IF(JA)150,150,155                                              C  007:004412
  145 LINE(101)=JZ                                                   C  007:004610
      GO TO 160                                                      C  007:004712
  150 LINE(1)=JZ                                                     C  007:004715
      GO TO 160                                                      C  007:004910
  155 LINE(JA)=JL(I)                                                 C  007:004913
  160 CONTINUE                                                       C  007:004B15
C  PRINT LINE OF DATA.                                               C  007:004E10
      IF(N/10=(N+1)/10)175,175,165                                   C  007:004E10
  165 WRITE(6,170)N,LINE,Y(1,N)                                      C  007:005014
  170 FORMAT(I4,I4,101A1,1X,1PF12.5)                                 C  007:005C12
                 *
ERROR # 11 "T" LEFT PARENTHESIS EXPECTED                                        (106)

      GO TO 185                                                      C  007:005C12
  175 WRITE(6,180)LINE,Y(1,N)                                        C  007:005C15
  180 FORMAT(5X,101A1,1X,F12.5)                                      C  007:006712
C  SET LINE VARIABLES TO ZERO.                                       C  007:006712
  185 DO 190 I=1,101                                                 C  007:006712
      LINE(I)=JBLANK                                                 C  007:006810
  190 CONTINUE                                                       C  007:006914
  195 N=N+1                                                          C  007:006B15
      IF(N-NF)110,110,200                                            C  007:006D11
  200 RETURN                                                         C  007:006E11
      END                                                            C  007:006F14
```