Title
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Publication Date
1970-08-01

Submitted to J. General Microbiology

UCRL-20046
Preprint

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August 1970

AEC Contract No. W-7405-eng-48

LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

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Kinetics of Ribosome Synthesis in Escherichia coli

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SUMMARY

A technique was developed for following the synthesis of ribosomal protein during the transition from one growth condition to another. By double-labelling the cells with [^{14}C]phenylalanine and [^{3}H]phenylalanine it was possible to measure both the cumulative ratio of ribosomal to total protein, and the differential rate of ribosomal protein synthesis during the transition from the lower to the higher growth rate. Shift-up experiments were carried out by adding glucose to cells growing in acetate or in succinate media. In both cases it was found that the differential rate of ribosomal protein synthesis rose slowly from its preshift rate to a max. 40-80 min. after the addition of glucose, and then declined. The cumulative ratio of ribosomal to total protein remained at its pre-glucose value for up to 40 min. after the shift, then slowly increased to a new higher value over the same time period as that in which the differential rate reached its max. These conclusions differ from those of Schleif (1967), who found that the differential rate of ribosomal protein synthesis rose, without overshoot, to its new value within 2-5 min.

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INTRODUCTION

While much progress has been made in elucidating the mechanism controlling the synthesis of individual proteins by examining the transcription and translation of particular genes, the mechanisms of the bacterial cell's gross control of the overall synthesis of protein and RNA have been more elusive. Because the ribosome is a key intermediary in the synthesis of all protein, it is hoped that the investigation of the control of ribosome synthesis will help explain the nature of gross control of the cellular economy. This study constitutes a series of measurements of the differential rates of ribosomal protein synthesis under conditions where the cellular economy is changed by shifting bacteria between differing growth media.

During steady-state growth all components of the bacterial cell increase at the same rate; the ratio of ribosomes to total protein thus remains constant. In bacteria growing at a constant temperature, but at different rates on different media, it has been found that the proportion of cellular protein present in the ribosomes is directly proportional to the growth rate (Schaechter, Maaløe & Kjeldgaard, 1962; Maaløe & Kjeldgaard, 1966).

In a shift-up from a relatively poor medium to a richer one, the cells must raise their ribosome level to that characteristic of the new medium. One of the most striking phenomena of such a shift is the uncoupling of the usually tight controls linking the rates of RNA and protein synthesis. RNA synthesis adjusts to its new differential rate in less than 1 min. (Maaløe & Kjeldgaard, 1966). In the case of shifts from a single carbon source to broth, there is an initial period

during which the rate of synthesis of total RNA is even higher than it will ultimately be in the new medium. Protein and DNA synthesis, on the other hand, accelerate much more slowly, usually continuing at the pre-shift rate for 10-25 min. after the introduction of broth, and then increasing to the new level characteristic of growth in broth. This dissociation has the effect of rather quickly raising the RNA/DNA ratio to that distinctive of the new growth conditions. On the other hand, to increase the rate of protein synthesis more ribosomes must be synthesized, unless there exists a significant number of inactive ribosomes.

In the present study we have investigated the kinetics of ribosomal protein synthesis during growth shifts. Such a study entails determining the kinetic ratios between two changing parameters, those of total protein synthesis and of ribosomal protein formation. Errors arising from either determination are compounded when a relationship between the two is sought. We have therefore extensively studied the factors affecting the reliability of measured changes in the kinetic ratio which result from the growth shifts.

METHODS

Organism. E. coli JC 14-2: alkaline phosphatase constitutive derivative of JC 14 (from Dr. A. J. Clark), obtained by the method of Torriani & Rothman (1961); $lac^+ade^-met^-$.

Growth. (a) Culture media. (i) Low phosphate-acetate (LP-acetate) medium contained: Tris-HCl, 100 mM; KH_2PO_4, 5 mM; NaCl, 80 mM; KCl, 20 mM; $(NH_4)_2SO_4$, 20 mM; $MgCl_2$, 1 mM; $CaCl_2$, 0.2 mM; Fe Versenol 120,

1.74 ml./l.; trace elements ($CuSO_4$.5 H_2O, 0.079 g./l.; H_3BO_3, 2.86 g./l.; $MnCl_2$.4 H_2O, 1.81 g./l.; $ZnSO_4$.7 H_2O, 0.22 g./l.; $CoCl_2$.6 H_2O, 0.04 g./l.; MoO_3, 0.015 g./l.), 1.0 ml./l.; adenine, 40 mg./l.; L-methionine, 100 mg./l.; thiamine, 0.5 mg./l.; Na acetate, 4 g./l. The medium was adjusted to pH 7.2. (ii) High phosphate-succinate medium (HP-succinate) was based on medium 63 (Pardee & Prestidge, 1961), containing Na succinate, 2 g./l., and supplemented with adenine, methionine and thiamine as above.

(b) Growth conditions: The cells were maintained continuously in liquid media for more than a year at 37°, with new sterile medium being inoculated every other day. The evening before an experiment, cells were inoculated from the stock culture into 50 ml. of fresh medium at a concentration too low to permit them to reach stationary phase overnight. The following morning the cells were stirred for 2-4 hr., and an appropriate aliquot diluted into approx. 200 ml. of the same pre-warmed medium in a 1 l. Erlenmeyer flask. Agitation was provided by means of a large teflon-coated bar magnet rapidly rotated by a magnetic stirrer below the flask, the latter being immersed in a constant temperature water bath at 37°.

For the experiments in which $^{14}CO_2$ was measured, 1 ml. of a log phase culture was transferred to the growth chamber described by Prevost & Moses (1967). Mixing and aeration were performed by bubbling a constant stream of moist air through the tube at a rate of 4-5 ml./min. (70 to 80 bubbles/min.).

(c) Growth measurements: Growth was followed by measuring E_{650} in a 1 cm. cuvette. Previous measurements in this laboratory (Moses &

Prevost, 1966) have shown that during balanced growth E_{650} is proportional both to total protein and to total RNA measured as total ribose. At E_{650} = 1.0, 1.0 ml. of culture contained 225 µg. of bacterial protein.

Labelling with radioactive precursors. The stock solution of -L-[G-^{3}H]phenylalanine contained 13.3 mM-amino acid at a specific radioactivity of 15 µCi./µmole. This stock solution was diluted 100-fold with the appropriate medium, a portion of which was used to grow the cells overnight; the rest was used for diluting the culture the following morning. In this way the phenylalanine in the cells became totally labelled with ^{3}H and the specific radioactivity was constant throughout the experiment. It can be calculated (Roberts et al., 1955) that at E_{650} = 1, E. coli will have taken up less than 10 µg./ml. of phenylalanine. Since the initial concn. of phenylalanine was greater than 20 µg./ml., and E_{650} never exceeded 1.0 during an experiment, less than half the phenylalanine was actually consumed.

During the course of the experiment L-[G-^{14}C]phenylalanine (specific radioactivity 400 µCi./µmole) was added undiluted to the growing bacteria, to give a concn. in a typical experiment of approx. 0.5 µCi./ml. The chemical concn. of phenylalanine was not appreciably affected by this addition, and the radiochemical ratio of ^{3}H to ^{14}C was approx. 4.1.

Measurement of total incorporation of amino acid. Samples (0.4 ml.) were removed from the culture vessel at intervals and added to preweighed tubes containing 0.2 ml. of chloramphenicol solution (1 mg./ml.) to prevent further protein synthesis. The tubes were weighed again,

permitting determination of sample sizes with an error of less than 1%. Trichloroacetic acid was then added (0.7 ml. of a 10% (w/v) solution), the contents of the tube mixed and allowed to stand for at least 30 min. at 0° before being filtered through a pre-wetted Millipore filter (0.45 μ pore size; Millipore Filter Corp., Bedford, Mass., U.S.A.), which retained the precipitated protein. The filter was washed repeatedly with trichloroacetic acid and water. After rough drying by sucking air through the filter, it was placed cells down in the bottom of a 20 ml. scintillation vial and moistened with 0.2 ml. of N-NaOH.

Comparison with untreated filters showed that the ^{3}H counts in NaOH-treated samples were both higher and more uniform among many vials in a series, perhaps because the protein particles were partially solubilized or broken into smaller fragments. This explanation assumes that ^{3}H is not counted accurately because the size of the protein particles was large enough to absorb some of the weak β-particles emitted from atoms inside the precipitated protein, an assumption we have not checked directly.

After 2 or 3 hr. at room temp., 18 ml. of scintillation solution (Kinard, 1957) was added and the vial agitated vigorously with a Vortex mixer to dissolve the filter pad. The scintillation solution was gelled by the addition of approx. 2% (w/v) Cab-O-Sil Thixotropic Gel Powder (Packard Instrument Co. Inc., Downers Grove, Illinois, U.S.A.) to keep the particles of protein in suspension. ^{3}H and ^{14}C were counted simultaneously with a Packard Tri-Carb scintillation counter, using external standardization. Corrections were made for ^{14}C counts appearing in the ^{3}H channel, and vice versa.

An experiment was performed in which growing bacteria were labelled with both [^{14}C]phenylalanine and [^{3}H]phenylalanine, and two parallel series of samples were taken, one processed with the NaOH treatment, and the other without. In both cases, the incorporated ^{14}C was linear with respect to extinction (Fig. 1). Incorporated ^{3}H in the untreated precipitates was linear with extinction at low cell densities, but departed from linearity as the cell density increased (Fig. 1); the NaOH-treated samples were linear with extinction over the entire range, although the slope of the

[Insert Fig. 1 near here]

line was significantly less than that for incorporated ^{14}C. According to the ^{14}C data, the bacteria incorporated 2.85 mg. of phenylalanine/100 mg. of protein synthesized. The comparable value from the ^{3}H data was 1.91 mg. Approx. this ratio of incorporation based on ^{14}C and ^{3}H determinations has been observed in many other experiments we have performed, including those in which uracil was used as a precursor for RNA.

The addition of glucose to the cells in the course of this experiment caused the growth rate to increase, as measured by E_{650}. However, optical extinction remained directly proportional to incorporated phenylalanine throughout the growth period (Fig. 1). Similar results have been obtained with L-[G-^{14}C]leucine and L-[G-^{3}H]leucine (J. Palmer, unpublished work).

Isotope measurements made in this way are designated total incorporation (T).

Analysis of the ribosome fraction. During each experiment, samples (approx. 4 ml.) were taken from the culture flask at intervals of

2-10 min. and placed in 20 ml. vials which had been pre-weighed and pre-cooled in liquid nitrogen. After quickly screwing on the top of the vial, it was swirled in liquid nitrogen for 15 sec. The entire sample was frozen within 25 sec. of its removal from the growth flask. These samples were stored at -20° until required. Each vial was reweighed to obtain an accurate sample wt., and thawed with the simultaneous addition of 2 ml. of cold solution No. 1 (see below), bringing the pH to 7.9. The cells were then disrupted for 5 min. at maximum power with a Bronwill "Biosonic" probe-type sonicator, keeping the contents of the vial below 4° in an ice-salt water bath. Sonicated suspension (5 ml.) was added to 1.0 ml. of cold solution No. 2 (see below) in a centrifuge tube, centrifuged for 15 min. at 10,000 x g average, and then for 60 min. at 34,000 x g average. In this and subsequent centrifugations a Spinco model L centrifuge was used with rotor No. 50. The supernatant from the last of these centrifugations was carefully pipetted into a propylene centrifuge tube and a sample (0.4 ml.) was removed. This sample was used to measure incorporated radioactivity in the supernatant protein. It is necessary to use polypropylene ("Poly-allomer") centrifuge tubes because the solution used subsequently to dissolve the ribosome pellet attacks cellulose nitrate tubes.

Before centrifuging again for 3 hr. at 100,000 x g average, enough unlabelled carrier ribosomes, previously isolated from E. coli by the procedure of Furano (1966), were added to give a final ribosome pellet about 3 mm. in diam. The addition of these carrier ribosomes also facilitated the sedimentation of the labelled ribosomes because

ribosomes dimerize at high concn., with a large increase in their s value (Peterman, 1964). The supernatant from the 100,000 x g centrifugation was discarded, the pellet resuspended in 6 ml. of standard buffer (see below) and left overnight at 0°in order to allow any free radioactive amino acid to equilibrate with the buffer. The next day it was again centrifuged at 100,000 x g average for 3 hr. and the supernatant discarded.

The bottom of each centrifuge tube around the pellet (a circle about 1 cm. in diam.), was excised, placed in a scintillation vial containing 2 ml. of NCS Solution Model 190620 (Nuclear Chicago Corp., Des Plaines, Illinois, U.S.A.), and shaken gently at 50° for 2 hr. to dissolve the protein (Hansen& Bush, 1967). Scintillation solution (18 ml.) containing 5 g. of PPO and 0.2 g. of POPOP/l. of toluene, was added, and radioactivity measured as before in a Tri-Carb liquid scintillation counter. Ribosomal protein is designated R.

The standard solutions used in ribosome isolation were as follows: Standard Buffer: Tris-HCl, 10 mM; Mg acetate, 10 mM; KCl, 50 mM; β-mercaptoethanol, 10 mM; L-phenylalanine, 0.6 mM; uracil (if required), 0.6 mM; pH adjusted to 7.8. Solution No. 1 contained β-mercaptoethanol, 0.15 ml.; L-phenylalanine, 0.8 mg.; uracil (if required), 0.4 mg.; N-NaOH, 70 ml. (or enough to bring 200 ml. of growth medium to pH 7.9); standard buffer, 30 ml. Solution No. 2 contained bovine serum albumin standard solution (10 mg. protein-N/ml.), 3 ml.; M-$MgCl_2$, 2.7 ml.; standard buffer, 24.3 ml.

Measurement of incorporation into the supernatant fraction. The protein in the supernatant fraction was precipitated by the addition of an

equal volume of 20% (w/v) perchloric acid. After 30 min. at room temp., each sample was filtered through a pre-wetted Millipore filter (0.22 μ pore size). The subsequent procedure was the same as described for the total incorporation samples. Better recovery of supernatant protein was obtained with perchloric acid as the precipitant, and a finer filter pad than used for whole bacteria. The labelled protein in this fraction was designated supernatant (S).

Method of analysis. In addition to measuring the two primary parameters (radioactivity incorporated into total protein and into ribosomal protein), we also measured label incorporated into the protein of the supernatant fraction from which the ribosomes were obtained by centrifugation. The radioactivity found in the supernatant protein was used to check the uniformity of cellular disruption by sonication, and as a general check on volumetric errors.

Use of the double-labelling approach permitted the acquisition of both long-term and short-term data. At the time of the growth shift, the cells had been growing for many generations in the presence of [^{3}H]phenylalanine, and this amino acid in the cellular protein was assumed to be essentially uniformly and completely labelled. The ^{3}H labelling could thus be used directly as a function of the amount of protein in each fraction studied. It was necessary only to divide the curve of label in ribosomal protein vs. time by the curve of label in total protein vs. time, at suitable intervals, to obtain the proportion of the total protein which was present in the ribosomes. The percent ribosomal protein obtained in this way was relative. We cannot be certain that our isolation of the ribosomes

succeeded in isolating all of them. Release of ribosomes from the cell during sonication may not have been complete, and/or ribosomes may have remained in the supernatant after the centrifugation at 100,000 x g. What does seem well established, as ascertained by the control experiments below, is that the fraction of the total ribosomes obtained by this technique was constant throughout an experiment, so that R/T measured the relative proportion of ribosomal protein in the cell as a function of time, and with respect to the growth shift.

The quantity which we were most interested in measuring was the differential rate of ribosomal protein synthesis,

$$\frac{\frac{d(\text{ribosomal protein})}{d(\text{time})}}{\frac{d(\text{total protein})}{d(\text{time})}} = \frac{dR}{dT} .$$

Because this measurement requires dividing the derivative of one curve by the derivative of another, the points defining the curves must be quite accurate. The results obtained from ^{3}H incorporation were not sufficiently precise, primarily because, during any particular time interval we used, the proportional change in the amount of incorporated ^{3}H would be very small. By adding [^{14}C]phenylalanine at the time of the growth shift it was possible to measure a rate of incorporation which began at zero, and which over each small time interval had a much larger relative change. From the [^{14}C]phenylalanine incorporated vs. time for total protein, ribosomal protein and supernatant protein one calculated dR/dT and dR/dS.

Even with this technique there were still difficulties in determining precisely the proper curve to be drawn through the incorporation points, because even slight differences in the curve would produce large changes in its derivative, and even larger changes in the ratio of two derivatives. A partial solution to this problem was obtained by using computer techniques to calculate the least squares best fit line through the data points, using a series of polynomials of the order 1 to 7 ($y = a_0 + a_1x + a_2x^2 + ... a_7x^7$). Since [^{14}C]phenylalanine incorporation was measured beginning immediately after its addition to the bacteria, the origin point (i.e. zero) was known absolutely, and this point was given 5 times the weight of any of the other points. Thus, there were 7 curves constructed for each set of data, and the problem was reduced to deciding which of the 7 was really the "best" fit. Orders 6 and 7 were always erratic, and their derivatives undulated in a way that indicated that they were changing within the inherent experimental error of each point. The derivative of the second order fit is a straight line, which is an artificial limitation on the differential rate of synthesis. The problem thus reduced to the choice between the third, fourth and fifth order curves, and several ratio calculations from each set of data could be made; those minor features of the resulting dR/dT curve which appeared in one order and not another could be attributed to an artifact of the plotting technique.

The variation among the curves as one went to higher order fit was also a good visual qualitative measure of the internal coherence of the data; the more the curves changed, the more scatter there was in the data.

Since the computer fitted the points to a single equation, the derivatives could easily be calculated at any desired intervals, and dR/dT determined as the ratio of the two derivatives at the same time. In the following experiments, results with both ^{3}H and ^{14}C labelling are presented, but it should be noted that those with ^{3}H are necessarily less precise than those with ^{14}C for the reasons discussed above.

Induction and assay of β-galactosidase. This followed previously established procedures (Palmer & Moses, 1968).

Measurement of $^{14}CO_2$ production. The method described by Prevost & Moses (1967) was used.

Chemicals and radiochemicals. L-[-^{14}C]Phenylalanine, L-[G-^{3}H]phenylalanine and [^{3}H]uracil were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [G-^{14}C]Glucose was prepared by the method of Putman & Hassid (1952). All other chemicals were from standard suppliers.

RESULTS

Control experiments

Purity of the ribosomes

Since ribosomes have a remarkable ability to bind proteins, particularly those with a net positive charge (Peterman, 1964), it was important to be sure that the ribosomes measured in these experiments did not contain large amounts of extraneous protein. The percentages of RNA and protein in the labelled ribosomes were determined by labelling the cells for a long period of time with [^{3}H]uracil and [^{14}C]phenylalanine, and processing replicate samples in the usual way.

Knowing the specific radioactivities of the labelled precursors, the percentage of uracil in the ribosomal RNA (6.75%, w/w) (Midgley, 1962; Spahr, 1962), and the percentage of phenylalanine in the ribosomal protein (4.01% w/w) (Spahr, 1962), it was possible to calculate the percentage of RNA and of protein in the ribosomal pellet. Six separate samples gave an average of 59 $\pm$ 1% RNA (w/w), compared with a reported value of 61% (Tissieres, Watson, Schlessinger & Hollingworth, 1959).

Measurements under steady state conditions

Before the changes in ribosomal protein synthesis due to the growth shift could be measured, it was necessary to test the analytical technique in the steady state condition. Cells in exponential growth in acetate medium, and cells in exponential growth in the same medium with the addition of 10^{-2} M-glucose for more than 10 generations, were sampled over the range of growth to be used in the growth shift experiments. Measurements were made of E_{650}, and samples were taken for total incorporation and for the preparation of ribosomes; these were processed as described in the Methods section. The night before the experiment began, [^{3}H]phenylalanine was added; [^{14}C]phenylalanine was added on the day of the experiment when E_{650} reached 0.3.

Fig. 2 shows a log plot of the phenylalanine incorporated into total protein (T), supernatant (S) and ribosomes (R) as measured by

[Insert Fig. 2 near here]

^{3}H counting, and E_{650} density for the acetate-grown cells. Fig. 3 shows the results obtained by dividing the ribosomal incorporation

[Insert Fig. 3 near here]

by the total or the supernatant (R/T or R/S), as well as supernatant divided by total incorporation (S/T), a measure of the uniformity of cell breakage. (The subscript numbers refer to the order of polynomial curve which was used to fit the points.) Ribosomal protein was 5.2 $\pm$ 0.2% of total protein and 17.2 $\pm$ 1.1% of the supernatant. The deviations from the horizontal line expected under steady state conditions were considerably less than $\pm$ 10% of the mean value, and probably represent close to an absolute minimum with this technique. Although the E_{650} was logarithmic throughout the experiment, at E_{650} = 0.45 the slope of the log ribosomal protein curve decreased sharply (Fig. 2). This was probably due to the cells beginning to depart from exponential growth, as they were then less than two generations from stationary phase. In this respect the ribosomes were more sensitive than light scattering as a measure of internal changes in the cell. R/T dropped sharply after this density, a warning that this kind of experiment must be conducted at rather low cell densities, E_{650} <0.4 for growth on acetate, and <0.8 for growth on glucose.

Supernatant protein was 31.5 $\pm$ 2.3% of total protein. This was lower than we usually found for the supernatant, but was uniform throughout the series of samples (less than 10% deviation from the mean). The variation found in S/T among our different experiments has not been satisfactorily explained, though most likely it was due to differences in the efficiency of sonic disruption. The sonication apparatus is tuned by ear, and although it was generally easy to keep it uniform for a given series of samples, this was not true for experiments done many months apart.

Similar results were obtained for cells growing on acetate plus glucose: the ribosomes accounted for 8.4 ± 1.3% of the total protein, an error of about 15%. In the growth shift experiments which follow, only changes considerably larger in magnitude than these will be considered significant. In spite of the experimental uncertainty, the mean values for R/T in acetate and glucose were in the expected ratio to one another. The ratio of the growth rates as determined optically was glucose/acetate, 1.58; the ratio of percent protein in ribosomes was glucose/acetate, 1.62. This agrees with the findings of Maaløe & Kjeldgaard (1966) that the number of ribosomes per cell mass is directly proportional to the growth rate.

In the control experiment, [^{14}C]phenylalanine was added to the acetate-grown cells when E_{650} reached 0.32; dR/dT and dR/dS were calculated from the curves for R, S, and T vs. time. The results confirmed those with [^{3}H]phenylalanine, with approximately similar degrees of error.

Growth shift experiments

Many growth shift experiments were performed, and while the degree of scatter of the experimental points varied on different occasions, the overall pattern of response was similar in each case. Two experiments have been selected for detailed presentation because in these there was a minimum of scatter and the results therefore have the greatest precision.

Shift from acetate to acetate plus glucose in low phosphate medium.

In LP-acetate medium the growth rate was 0.50 doublings/hr. After the addition of glucose this rate remained unchanged for 20 min., and

then shifted to 0.76 doublings/hr. There was a further slight increase in the growth rate to 0.80 doublings/hr. about 75 min. after the glucose was added (Fig. 4). Fig. 4 also shows the incorporation of [^{3}H]phenylalanine into total, ribosomal and supernatant protein. The

[Insert Fig. 4 near here]

curve for total protein shows the same discontinuity as the E_{650} curve: a sharp increase 25 min. after the addition of glucose. Ribosomal protein as a function of either total or supernatant protein rose steadily for 120 min., after which it appeared to be reaching a plateau.

Fig. 5 shows the incorporation of [^{14}C]phenylalanine into the total, ribosomal and supernatant protein fractions. From these was derived the differential rate of ribosomal protein synthesis, shown

[Insert Fig. 5 near here]

in Fig. 6. Accurate counting data for ^{14}C incorporation was impossible to obtain for the first few min. after the addition of

[Insert Fig. 6 near here]

[^{14}C]phenylalanine because of the very high ^{3}H/^{14}C ratio at that time. Data for the first 15 min. after the growth shift have therefore been omitted from Fig. 6. By plotting in one graph curves obtained with three different polynomials one can see the differences and similarities produced by different methods of calculation. When there are differences we can only assume a state of ambiguity. However, all three curves agree in showing that the differential rate of ribosomal protein synthesis began to rise 20-45 min. after the addition of glucose, that it reached its peak value after 85-100 min., and

thereafter declined sharply; it was still falling at the end of the experiment (130 min. after glucose was added).

Although there were significant differences in the quantitative values obtained by the use of different fractions and methods of calculation, qualitatively all methods showed that the response to glucose was slow, that the bacteria were increasing their ribosomal protein content over the period 20-100 min. after the addition of glucose, and that there was an overshoot in the differential rate of ribosomal protein synthesis. This means that the bacteria synthesized ribosomal protein at a rate higher than that necessary for the new steady state condition; i.e., for a measurable period there was a preferential synthesis of ribosomal protein at the expense of other cellular protein.

In an independent repetition of this experiment essentially the same result was seen: dR/dT and dR/dS began to increase soon after glucose was added, reached a maximum 55-80 min. later, and then declined sharply.

Shift from succinate to succinate plus glucose in high phosphate medium

With succinate as sole carbon source in high phosphate medium, strain JC 14-2 grew at the rate of 0.94 doublings/hr. Upon addition of glucose, the growth as measured by E_{650} showed an immediate increase to 1.11 doublings/hr. A further increase in growth rate (to 1.33 doublings/hr.) occurred 12 min. after the addition of glucose, and 35 min. later the growth rate reverted to 1.11 doublings/hr. (Fig. 7). The growth rate as revealed by [^{3}H]phenylalanine labelling showed an increase of roughly the same magnitude, but the scatter of these

determinations did not permit divergencies over short periods of time to be detected (Fig. 7).

[Insert Fig. 7 near here]

Although an increase in the rate of synthesis of ribosomal protein was observed immediately after the growth shift, there was a sharp increase in the differential rate of synthesis starting 40 min. after the addition of glucose, reaching a maximum after 90 min. (Fig. 8). Variations in the differential rate before the increase showed a deviation of little more than 10% of the mean, and fall within established error limits.

[Insert Fig. 8 near here]

Calculation of the differential rate of ribosomal protein synthesis using the data from [^{14}C]phenylalanine labelling confirmed this general pattern, with the increase starting at about 20 min. after glucose was added, a maximum at 85 min., and a fall towards 120 min. (Fig. 9). Curves drawn using higher order polynomials showed essentially the same kinetics, though with greater undulation. It is

[Insert Fig. 9 near here]

clear from this experiment, as from the one in which cells were shifted from acetate to glucose, that the response of the cells to glucose was slow, requiring more than one generation time for the maximal rate of ribosomal protein synthesis to develop.

Other measurements of the rapidity of the response to glucose

A possible reason for the persistent lag in the response of ribosomal protein synthesis to the introduction of glucose might have been a delay in the onset of glucose metabolism. Two independent tests of glucose metabolism were therefore made.

The addition of glucose to E. coli induced to synthesize β-galactosidase typically produces a rapid intense transient repression of enzyme synthesis (Moses & Prevost, 1966). This was found to hold for cells growing in LP-acetate medium on the addition of glucose (Fig. 10). Repression became apparent within about 3 min. of introducing glucose.

[Insert Fig. 10 near here]

The release of $^{14}CO_2$ from [G-^{14}C]glucose began within 2 min. of adding labelled glucose to cells in low phosphate medium containing either acetate or acetate plus unlabelled glucose (Fig. 11). Since it had previously been established (Prevost & Moses, 1967) that there was a delay of 1 min. between the release of $^{14}CO_2$ in the growth

[Insert Fig. 11 near here]

chamber and its detection in the scintillation flow counter, the maximum delay in the onset of glucose utilization was 1 min., and this could not account for the lag in the increase in the differential rate of ribosome synthesis.

DISCUSSION

It is possible, by the techniques described here, to measure kinetically the differential rate of ribosomal protein synthesis. Before useful data could be obtained it was necessary to study the errors and limitations of the method, and to work out experimental and mathematical criteria for assessing the significance of the findings.

It proved impossible to standardize the efficiency of cellular disruption, and thus permit absolute comparisons between experiments.

Nevertheless, the experimental points did allow the kinetics of ribosome synthesis to be observed when all the measurements were made on one culture. Thus, while we have no reliable absolute measure of the differential rate of ribosome synthesis, we were able to follow relative changes of this parameter in growth shifts.

One possibility requiring consideration was that we were labelling nascent protein attached to ribosomes, rather than ribosomal protein itself. A number of arguments eliminate this possibility. The time required for E. coli to synthesize a protein molecule de novo is not more than 3 min. (Branscomb & Stuart, 1968), while the internal pool of amino acids is sufficient for a few seconds of growth only (Britten & McClure, 1962). One would therefore expect the specific radioactivity of the nascent protein effectively to reach that of the labelled precursor within 3 min. Thereafter, total label in nascent protein could increase only as the population of ribosomes increased. This should have been seen experimentally as an initial rapid labelling of the ribosome fraction, followed by a slow rise. No such kinetic behaviour was observed (Fig. 5). Schleif (1967) labelled ribosomes in vivo by a pulse-chase method using labelled proline. He found similar amounts of label incorporated into the ribosome fraction when the chase period was 2.5 min. or 40 min. Thus, a 2.5 min. chase was long enough to permit incorporation of labelled ribosomal precursors into ribosomal protein, and also to flush out label from nascent protein. Further, it has been found in this laboratory that measurement of the differential rate of ribosome synthesis by the incorporation of labelled

uracil into ribosomal RNA yielded similar kinetics in growth shifts to those obtained with phenylalanine labelling (H. G. Ungar, unpublished work).

Label in nascent protein could only have been of significance in our experiments during the early stages of ^{14}C labelling; with ^{3}H labelling, the cells were totally labelled, so that label in nascent protein must have formed a small part of all the label in the ribosomal fraction. Bacterial ribosomes have a mol. wt. of about 2.7×10^6, of which 39% is protein (Tissieres et al., 1959); the ribosomal protein thus contributes about 1.06×10^6 daltons. If we assume that all ribosomes are engaged in the synthesis of protein subunits, of average mol. wt. 30,000, then the average ribosome will be attached to a half-completed polypeptide of mol. wt. 15,000. Thus, about 98.5% of all the protein in the ribosomes should be ribosomal protein. Our experimental data (Fig. 8 and 9) indicate similar conclusions based on ^{3}H and ^{14}C data, and we infer that since the ^{3}H data refers overwhelmingly to ribosomal protein, the ^{14}C data does so as well.

In all of the growth shifts studied there was a very similar pattern. The differential rate of ribosomal protein synthesis increased slowly in response to the addition of glucose, requiring a generation or more to reach its maximum rate. This maximum was higher than the rate necessary for the new growth conditions, and eventually the rate began to fall from its maximum value. In addition to the detailed experiments reported here, a number of others were performed, including replicates of those discussed

and shifts from acetate to glucose in high-phosphate medium; all showed a similar kinetic pattern of ribosome synthesis. Of the two findings, the delayed response is perhaps more firmly established than the overshoot. To have followed the differential rate of ribosome synthesis until it settled down at a rate characteristic of the new growth medium required samples to be taken for several hr. after the shift. Exponential growth ceased, however, before sufficient time had elapsed. Further, to observe the whole process proved difficult because of the inaccuracy of radioactivity measurements with unfavourable ratios of ^{3}H and ^{14}C. In order to obtain reasonable accuracy for the ^{14}C counting at the beginning of the experiment, the specific radioactivity of [^{14}C]phenylalanine needed to be relatively high. This very fact then produced a very unfavourable counting ratio later on, as the proportionate rate of increase of ^{14}C counts was much higher than that of ^{3}H counts. It was thus possible to observe that the differential rate of ribosomal protein synthesis passed through a maximum, but not to obtain the new steady rate without either diluting the culture, or using two parallel flasks, to one of which [^{14}C]phenylalanine was added much later than the other. When the latter approach was explored it was found that discrepancies between the parallel flasks precluded a direct comparison between them.

Although a rise in the differential rate of ribosome synthesis was invariably delayed, this was not the case with total protein synthesis. Fig. 6 shows that, in cells shifted from succinate

to succinate plus glucose, there was an immediate increase in the rate of protein synthesis. Many experiments over the years in this laboratory have shown that immediate increases in the rate of protein synthesis (as measured by E_{650} or by labelled amino acid incorporation) follow enrichment of the medium. This can be very large: a shift from acetate to broth may result in a threefold increase in the growth rate within 1 or 2 min. Such increases suggest the existence of unused ribosomal capacity for protein synthesis, and the same conclusion may be drawn from the present results.

In order to bring the ribosome content up to the new level characteristic for the faster growth rate, the cells must either make ribosomes preferentially for a period, or only gradually accumulate the required ribosomal content. An overshoot is there not unexpected. While we have as yet no experimental data on the factors controlling ribosome synthesis, we surmise that they are made in response to metabolic signals. Enrichment of the medium would presumably alter the rate of ribosome synthesis. Growth shifts, however, are not accomplished quickly (Moses & Sharp, 197), and regulatory phenomena are known which are particularly evident during the metabolic rearrangement which occurs then (Moses & Prevost, 1966; Prevost & Moses, 1967). It seems likely that just as transient repression of catabolic enzymes takes place during growth shifts, so transient enhancement of ribosomal protein synthesis also

occurs. It remains to be seen to what extent these various transient phenomena can be correlated.

Schleif (1967) studied growth shifts in E. coli B/r. His technique differed from ours in that he used a pulse-chase method of labelling (exposure to labelled proline for 1 min., followed by unlabelled proline for 40 min.) before isolating the ribosomes. He showed that a chase period of less than 5 min. was enough to permit incorporation of the labelled proline into ribosomal protein, with no further appearance of label in ribosomes when the chase was prolonged to 40 min. From his result, Schleif calculated that the pool of pre-ribosomal protein would suffice for a maximum growth period of 5 min. (his experimental data suggest a period of not more than 2.5 min.). The application of this conclusion to our results would shift our kinetics by not more than 2.5 - 5 min.

Schleif's overall conclusion clearly differs from ours: he reported no overshoot in the differential rate of ribosomal protein synthesis during a growth shift, and attainment of the new differential rate within 2 - 5 min. of adding glucose to cells in succinate-minimal medium. However, examination of his published data casts doubt on this conclusion. His graph (Fig. 8 in his paper) of the differential rate of ribosomal protein synthesis reports two experiments. In one of them the first two experimental points after the growth shift were at 2 min. and 25 min., and the conclusion that the differential rate

reached its maximum value after 2 min. depends entirely on that first point. In the second experiment of his Fig. 8, the differential rate reached a maximum value 16 min. after the shift, with a slight decline in rate at longer periods for both experiments.

Schleif also presented comparative data for the total cellular protein (measured chemically) and the total acid-soluble ^{32}P (from $^{32}P_i$), which he took to be a measure of ribosomal RNA synthesis (Fig. 9 in his paper). Assuming, therefore, that incorporated ^{32}P is proportional to ribosomal protein, we have calculated from Schleif's graphs the differential rate of ribosomal protein synthesis following the addition of glucose. The result shows an increase in this parameter starting at the time of the growth shift, passing through a shallow maximum 40-50 min. later, declining to a trough at 70-80 min., and continuing to rise until the end of the measurements at about 125 min. (Fig. 12). These values are subject to some uncertainty, taken as they were from the printed graphs of Schleif's paper.

(Insert Fig. 12 near here]

We therefore consider that, while the onset in the increase in the differential rate of ribosome synthesis was delayed in our system compared with Schleif's, there is no real disagreement between his experimental results and ours on the slow attainment of the final differential rate, and the existence of an overshoot.

The work reported in this paper was sponsored by the U.S. Atomic Energy Commission. We acknowledge with thanks valuable discussions with Dr. O. Maaløe and Dr. J. Gerhart during the early part of this work, and technical assistance from Miss Pamela B. Sharp with some of the experiments.

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Running title: Kinetics of ribosomes synthesis in E. coli

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Captions for figures

Fig. 1. Effect of NaOH treatment on ^3H counting. A mixture of [G-^3H] phenylalanine and [G-^{14}C] phenylalanine was added to cells growing exponentially in LP-acetate medium. Glucose (10 mM) was added at arrow. Two parallel series of samples were precipitated with trichloroacetic acid and collected on Millipore filters. O, ^3H incorporated, no NaOH treatment; ●, ^3H incorporated, 2 hr. NaOH treatment; ▲, ^{14}C incorporated, with or without NaOH treatment.

Fig. 2. [G-^3H] phenylalanine incorporation during exponential growth on acetate. Incorporation into total protein (T), supernatant protein (S) and ribosomal protein (R) shown, together with E_{650}.

Fig. 3. Relative ribosome content during balanced growth on acetate; based on ^3H data. A, R_3T_3; B, R_3/S_3; C, S_3/T_3; subscript numbers indicate order of polynomial used in calculations. Same experiment as that reported in Fig. 2.

Fig. 4. Growth shift in LP medium from acetate to acetate plus glucose. Glucose (10 mM) added at 0 min. Based on 3_H data. T, ^3H in total protein; S, ^3H in supernatant protein; R, ^3H in ribosomal protein; E_{650} as indicated. The three times periods A, B and C indicate different growth rates as determined by E_{650}: A, 0.50 doublings/hr.; B, 0.76 doublings/hr.; C, 0.80 doublings/hr.

Fig. 5. Kinetics of [^{14}C] phenylalanine incorporation during the transition in LP medium from acetate to acetate plus glucose; same experiment as Fig. 4. Glucose (10 mM) added at 0 min. A, total protein; B, ribosomal protein; C, supernatant protein. Full lines indicate 4th order polynomial curves; dashed lines indicate 5th order polynomial curves.

Fig. 6. Differential rate of ribsomal protein synthesis, as calculated from ^{14}C incorporation data, in the same experiment as that reported in Fig.4. A, dR_4/dT_4; B, dR_5/dT_5; C, dR_4/dS_4: subscript numbers indicate order of polynomial used in calculations. [G-^{14}C] phenylalanine added together with glucose at 0 time.

Fig. 7. Growth shift in HP medium from succinate to succinate plus glucose. Glucose (10 mM) added at 0 min. Based on ^3H data. T, ^3H in total protein, S, ^3H in supernatant protein; R, ^3H in ribosomal protein; E_{650} as indicated.

Fig. 8. Relative ribosomal content, as calculated from ^3H data, in the same experiment as that reported in Fig. 7. Glucose added at 0 time. Curve shows R_7/T_5; subscript numbers indicate order of polynomial used in calculation.

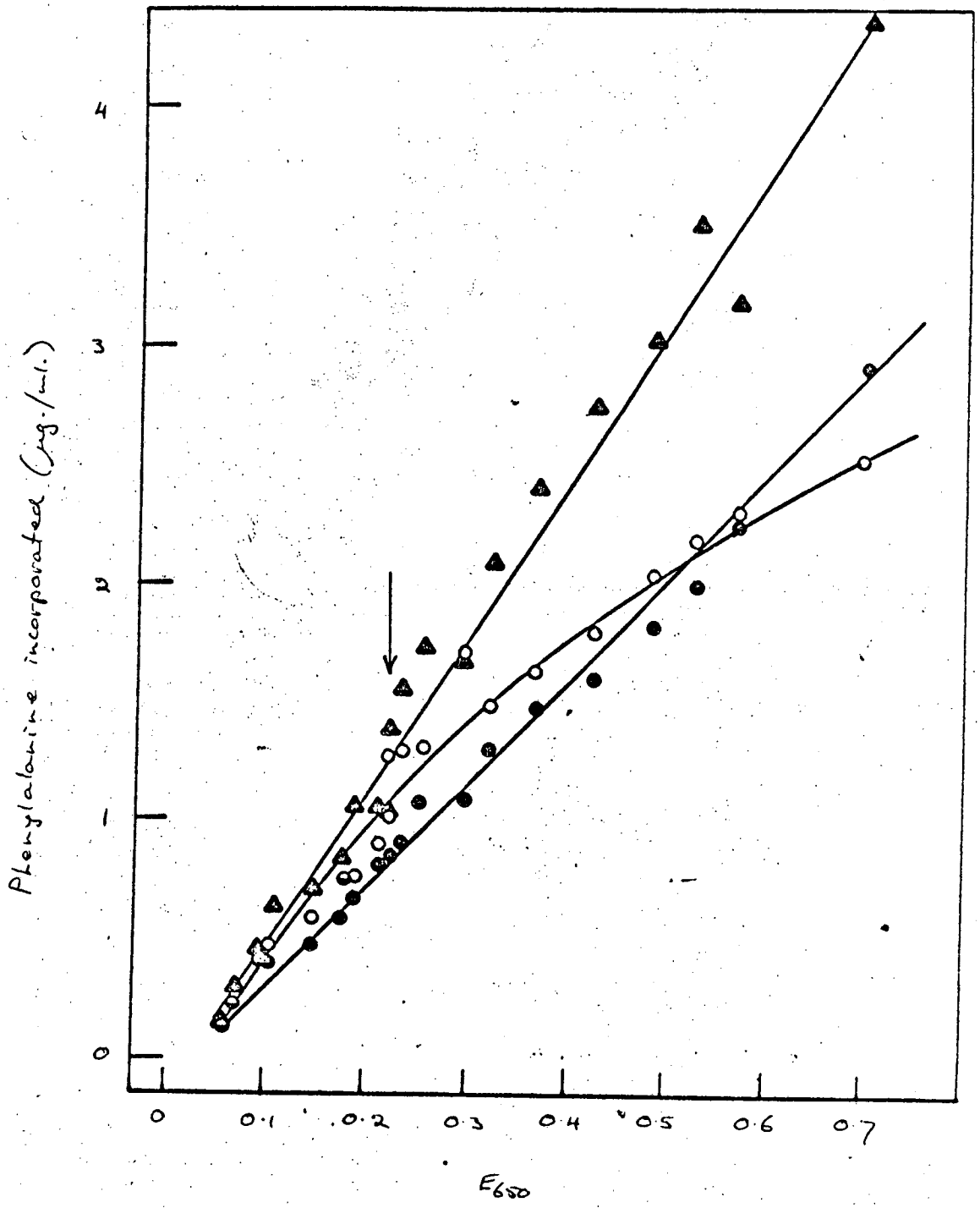
Fig. 9. Differential rate of ribosomal protein synthesis, as calculated from ^{14}C incorporation data in the same experiment as that reported in Fig. 7. Curve shows dR_4/dT_4; subscript numbers indicate order of polynomial used in calculation. Glucose added together with [G-^{14}C] phenylalanine at 0 time.

Fig. 10. Transient catabolite repression of β-galactosidase synthesis. Glucose (10 mM) added to cells growing exponentially in LP-acetate medium at 0 time. A, β-galactosidase; B, growth.

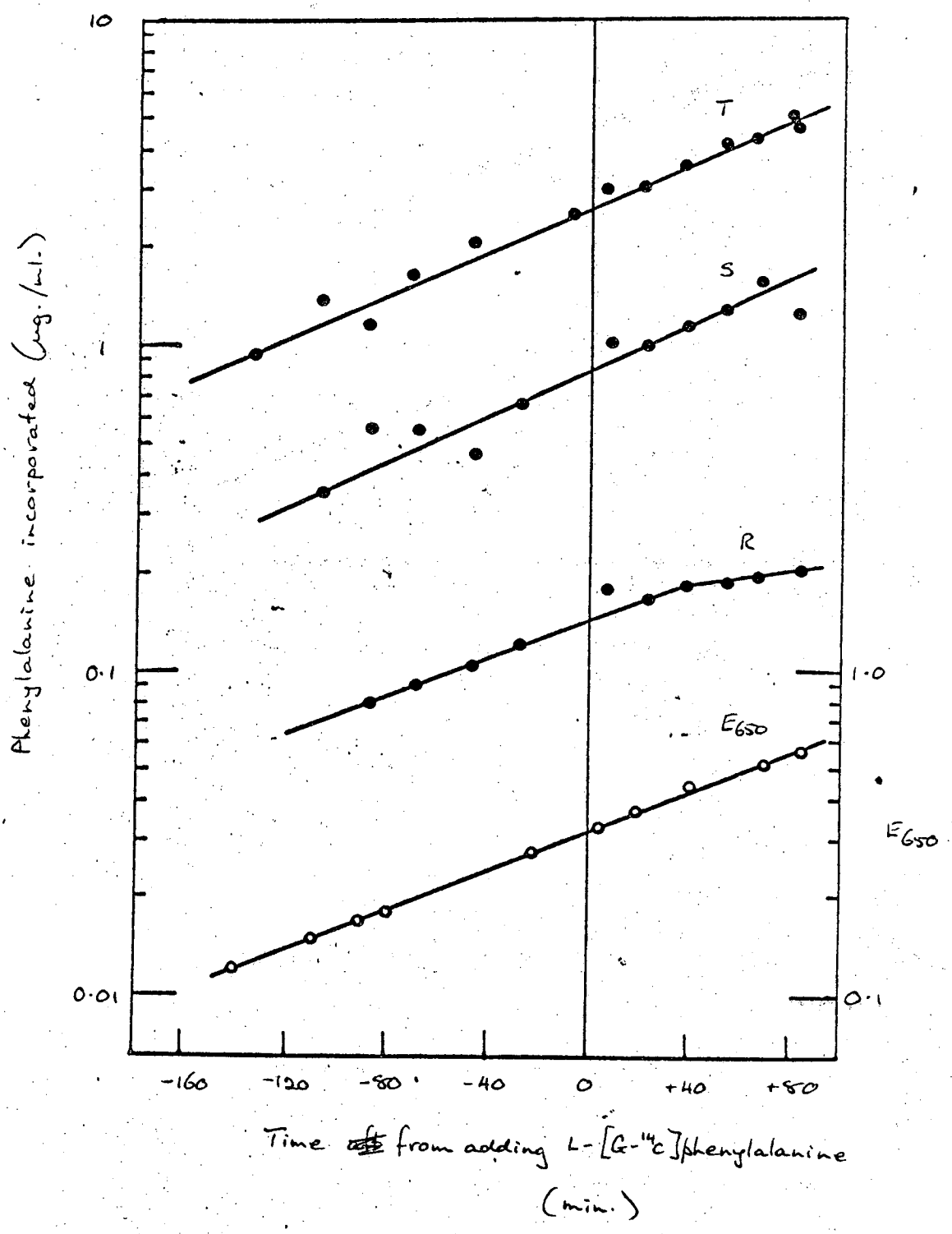
Fig. 11. Production of $^{14}CO_2$ from [G-^{14}C] glucose by exponentially growing cells. A, cells growing in LP-acetate: [G-^{14}C]glucose (10 mM) added at 0 time; B, cells growing in LP-acetate containing 10 mM glucose: [G-^{14}C]-glucose added at 0 time with no significant change in total glucose concn.

Fig. 12. Differential rate of ribosome synthesis calculated from the data of Schleif (1967: Fig. 9). Glucose was added to cells of E. coli B/r in succinate-minimal medium at 0 min. Total protein was measured chemically, and ribosomal RNA with ^{32}P. Following Schleif, we have assumed that ribosomal RNA is proportional to ribosomal protein.

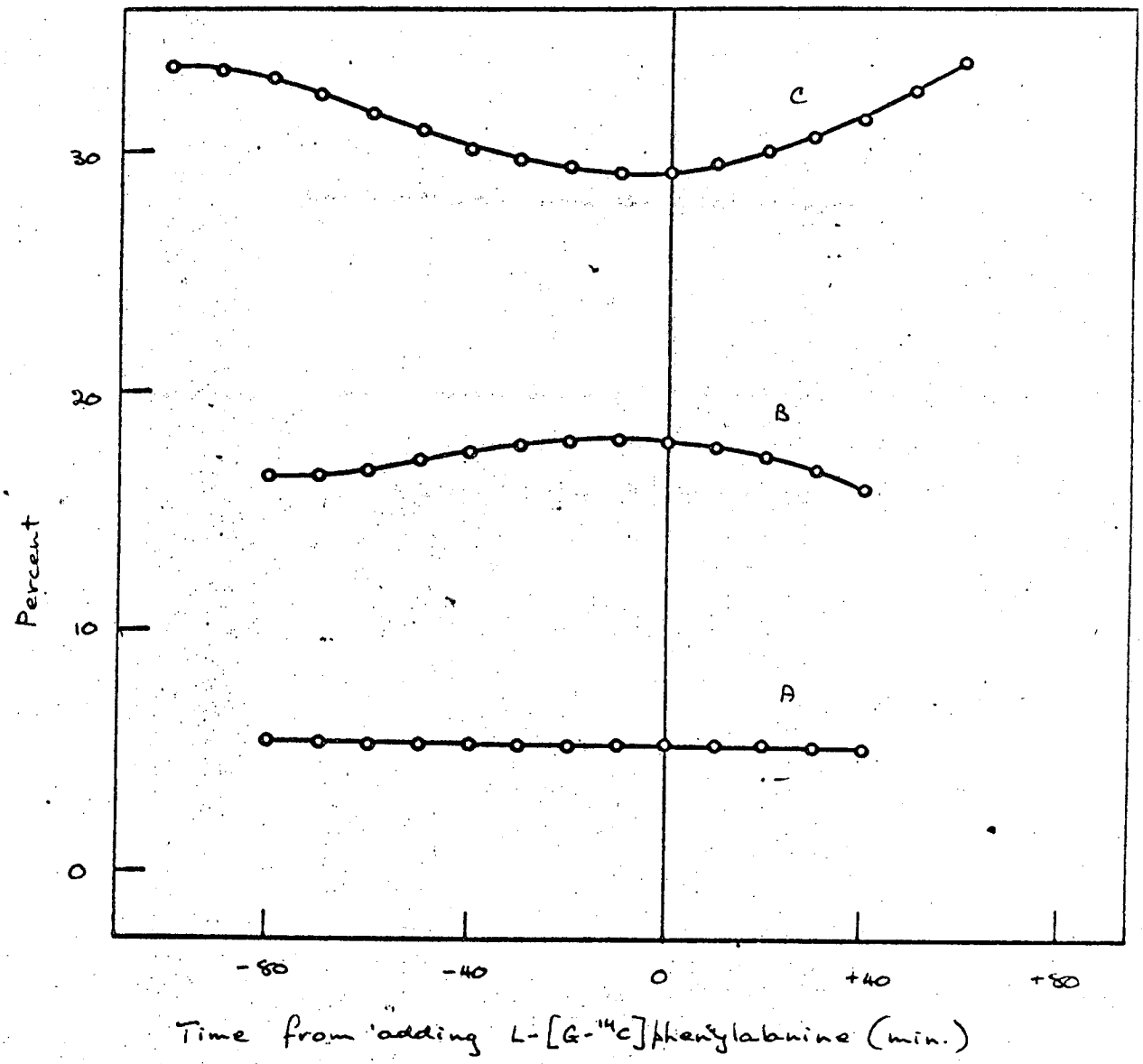
UNGAR & MOSES. FIG. 1



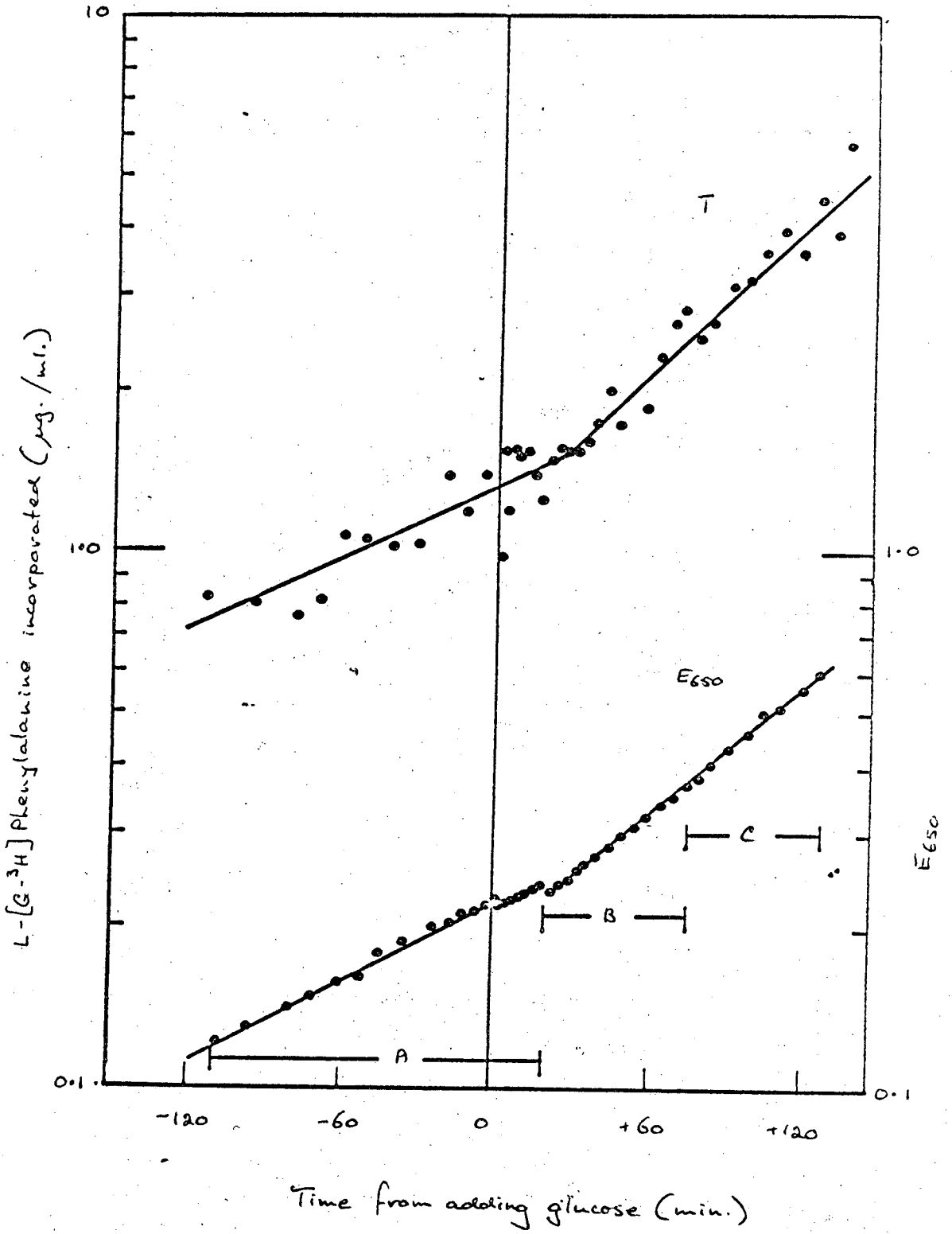
UNGAR + MOSES (FIG. 2).

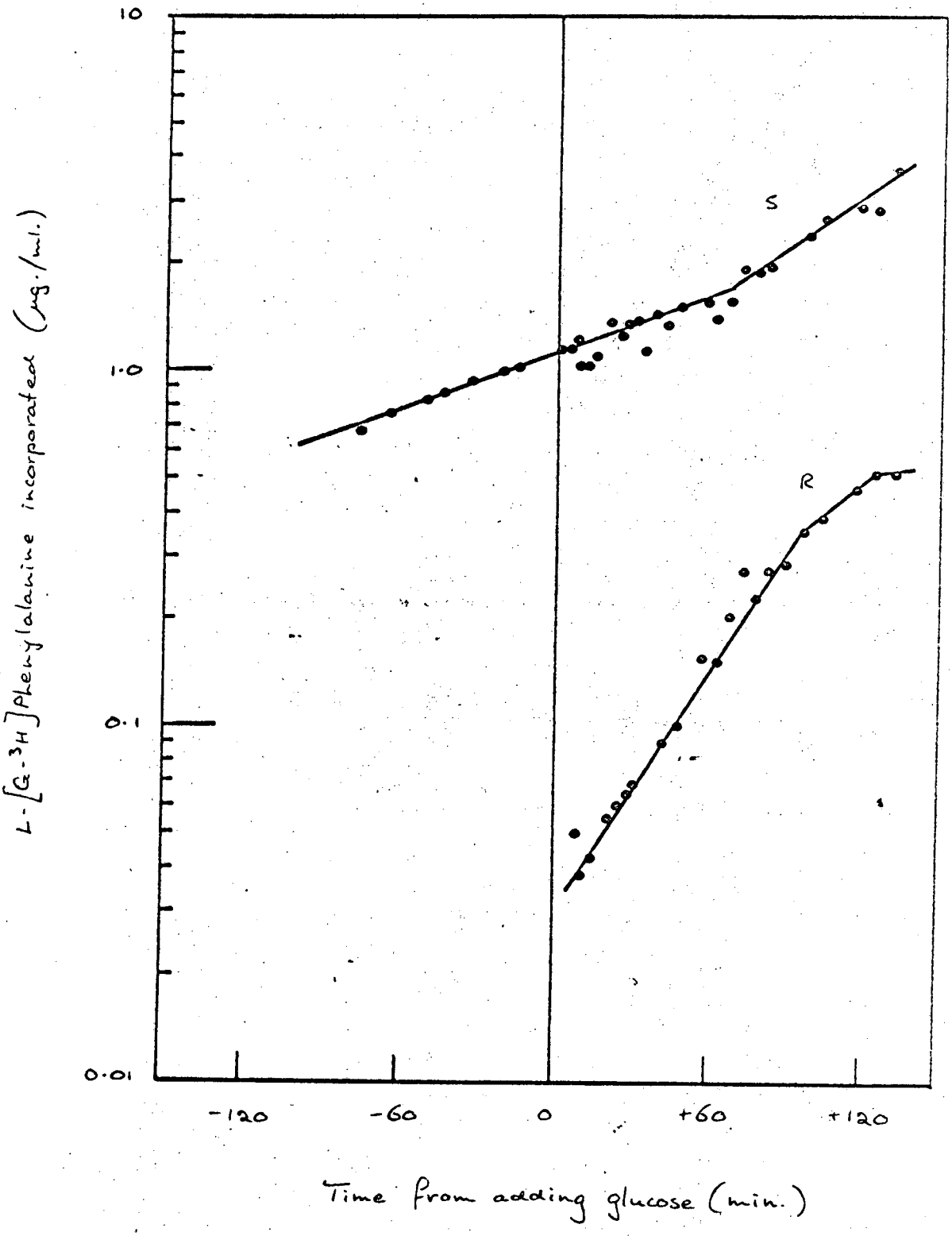


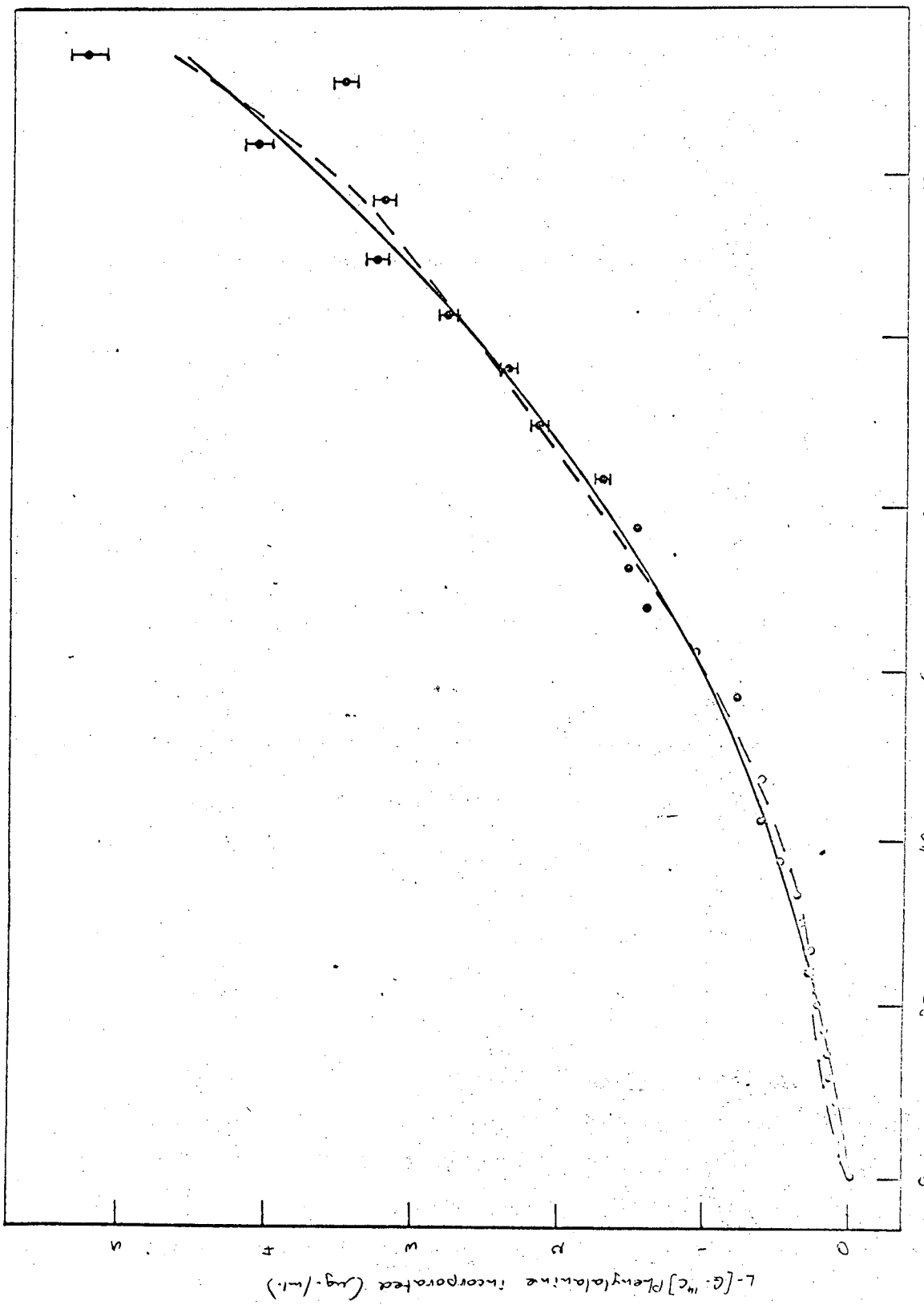
UNGAR & MOSES (FIG. 3).

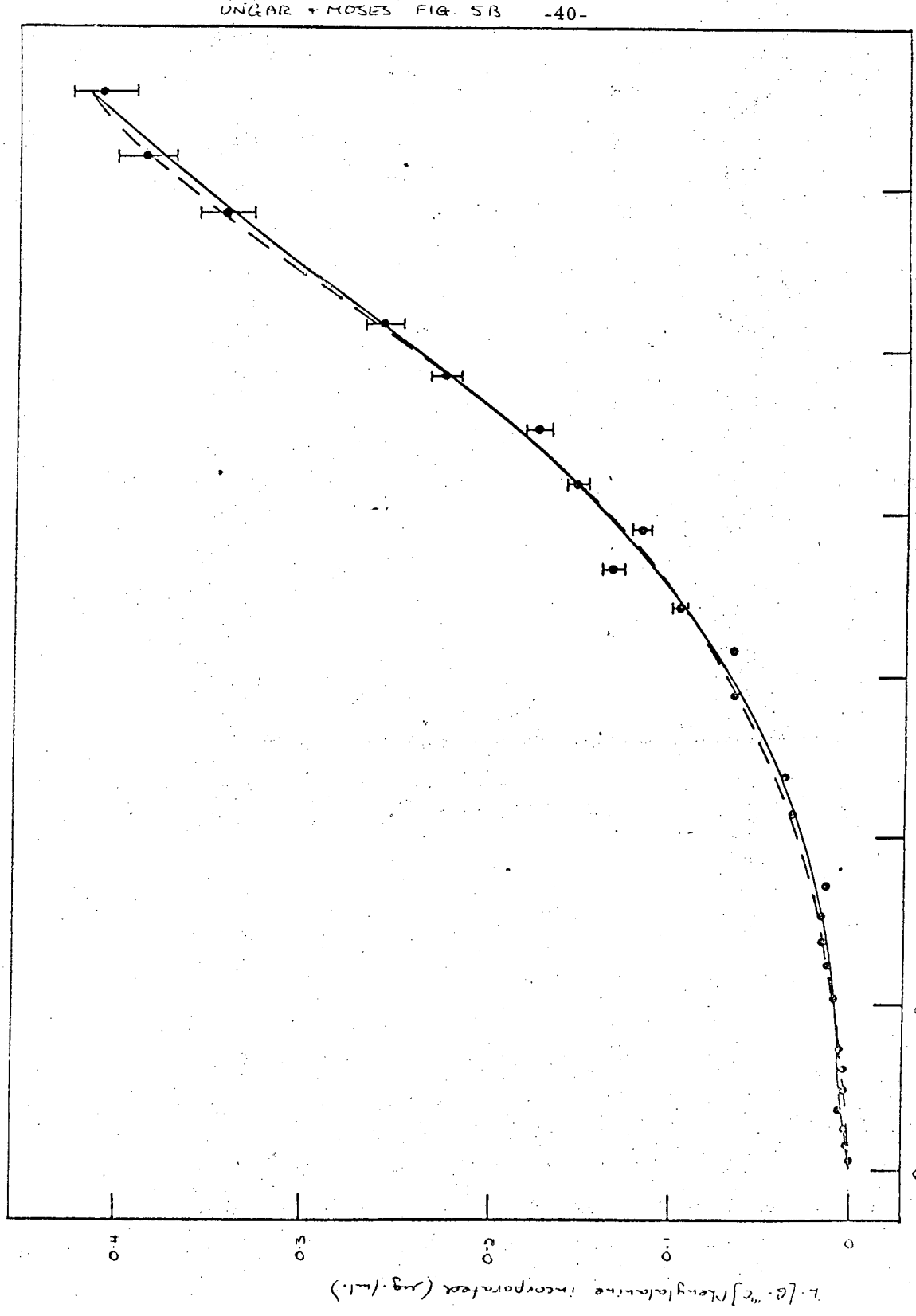


UNGAR & MOSES. FIG. 4 (TOP).

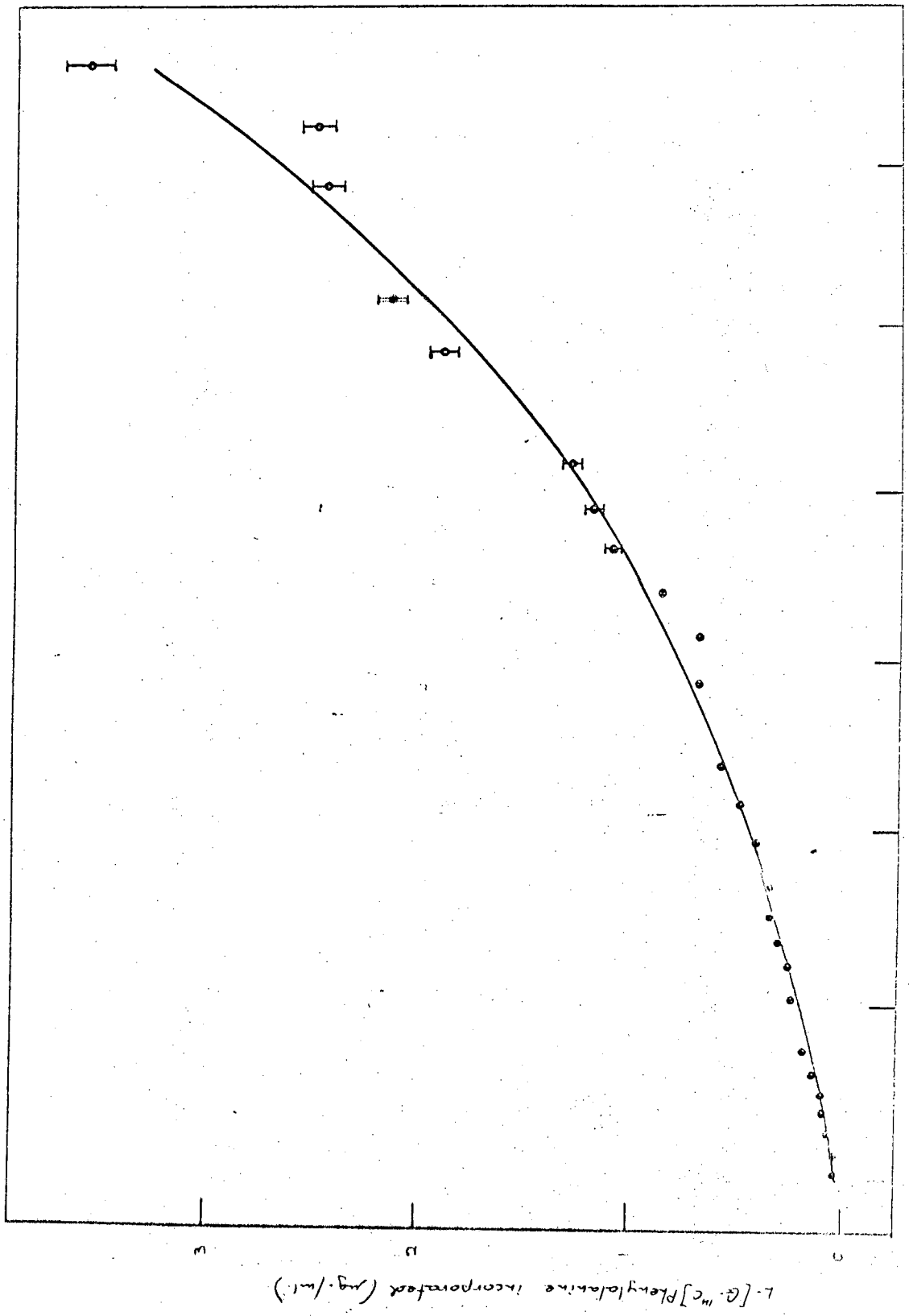



10
1·0
0·1
0·01
S
R
-120
-60
0
+60
+120
L-[G-3H]Phenylalanine incorporated (mg./ml.)
Time from adding glucose (min.)

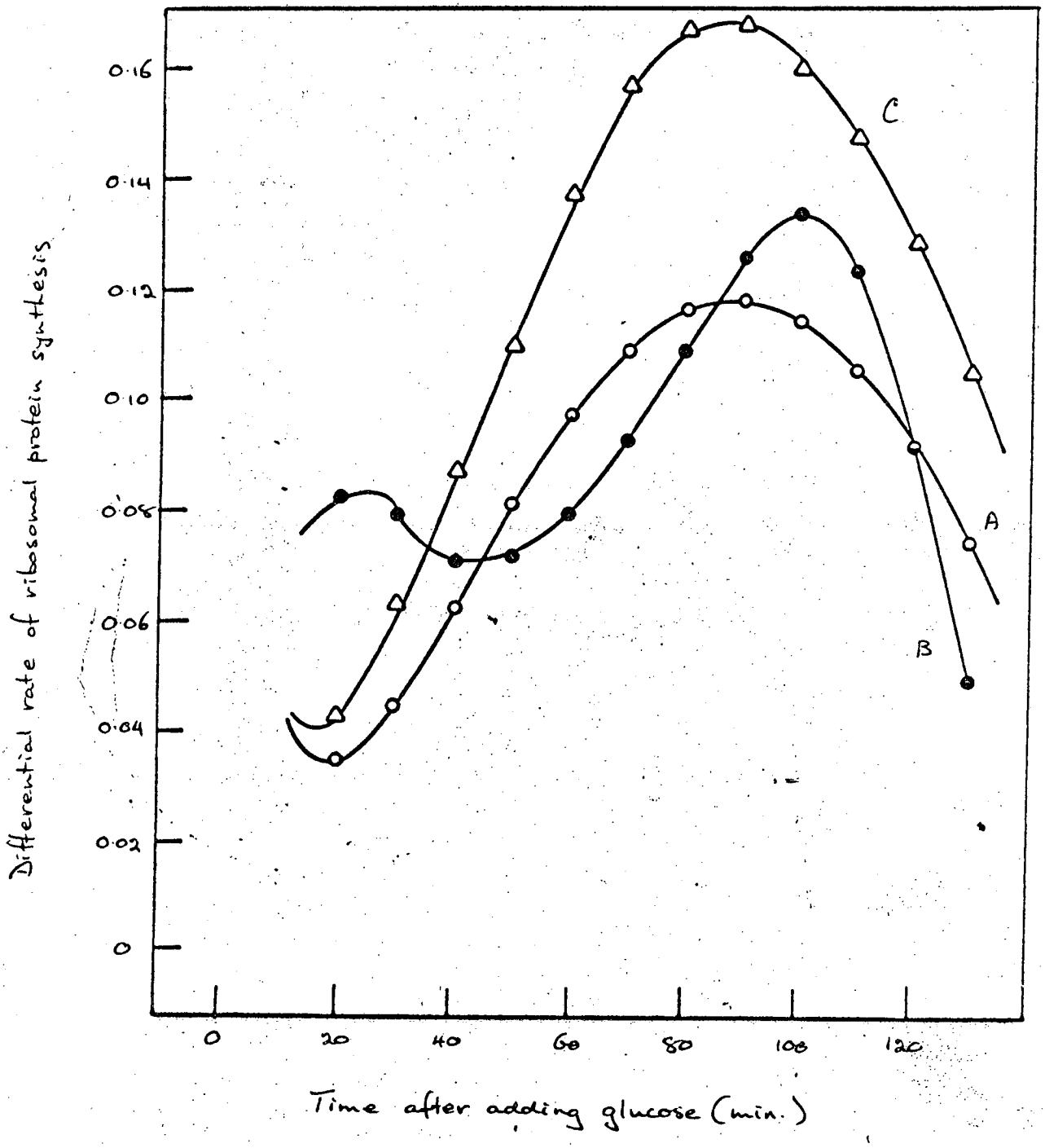

L-[G-14C] Phenylalanine incorporated (μg-/ml.)
0
40
80
120
160
200
0
20
40
60
80
100
120
Time after adding glucose (min.)


L-[G-14C]Phenylalanine incorporated (mg./ml.)
0
0.1
0.2
0.3
0.4
0
20
40
60
80
100
120
Time after entering glucose (min.)

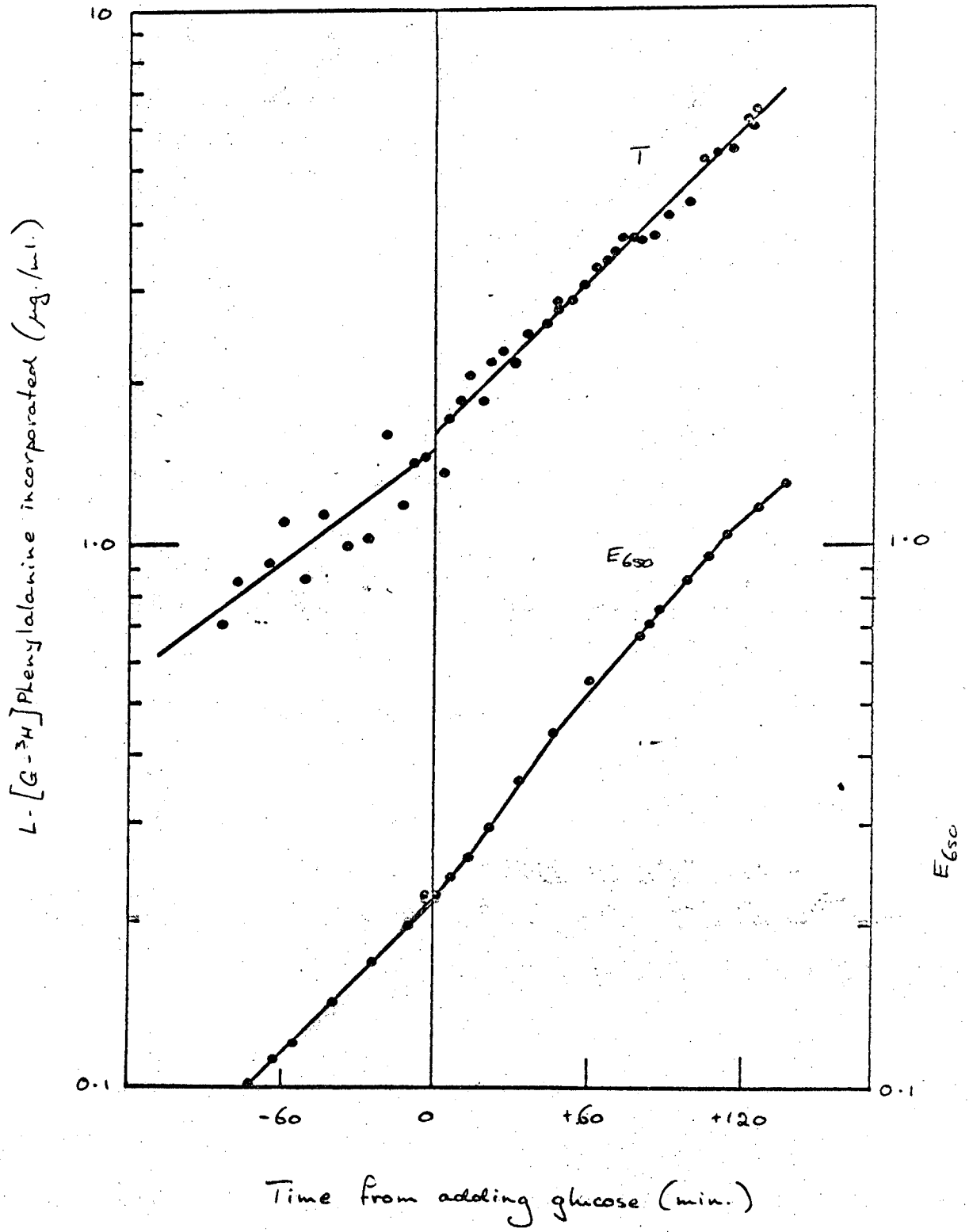
UNGAR + MOSLEY FIG. 5C.



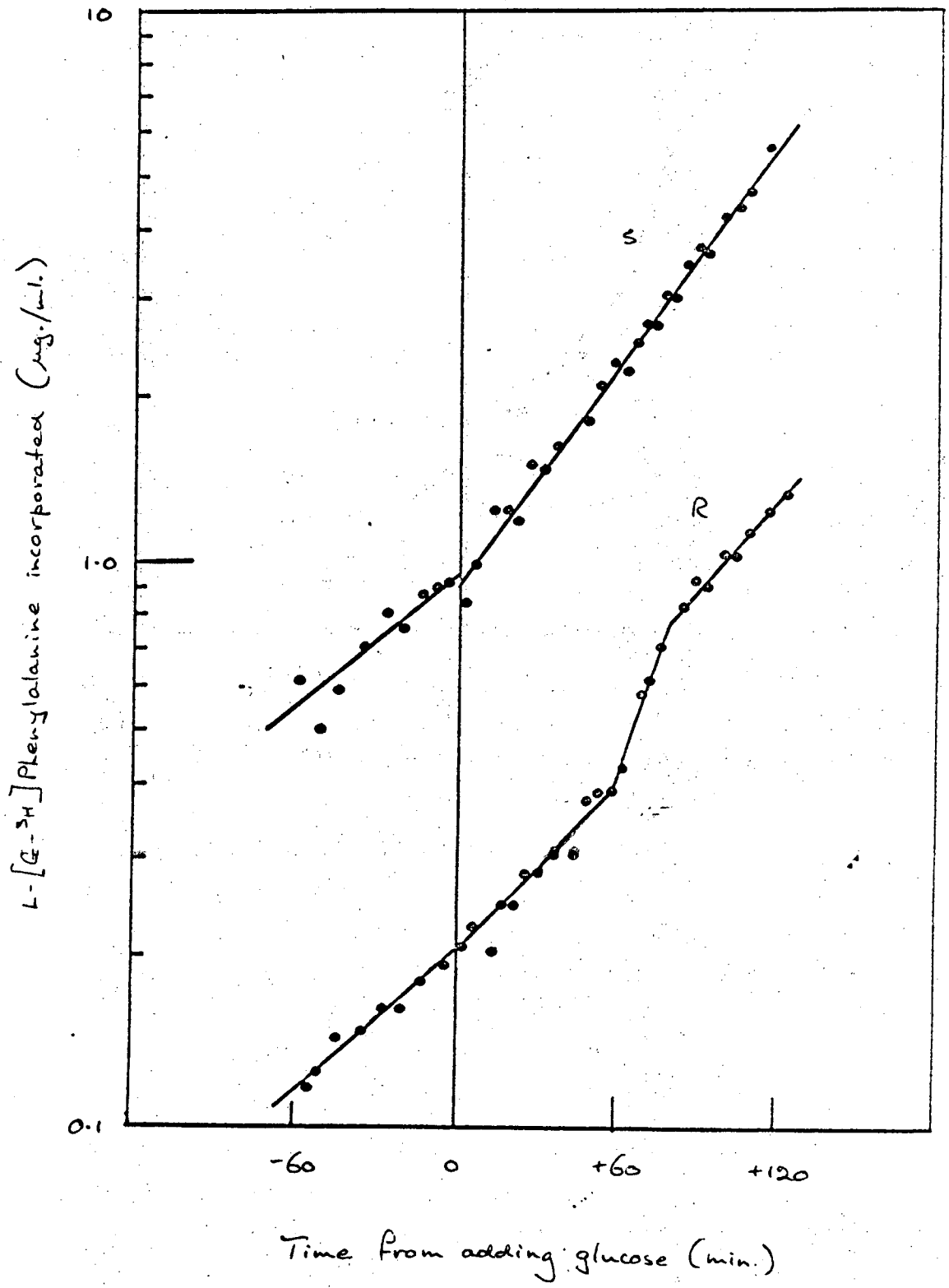
UNGAR & MOSES FIG. 6.



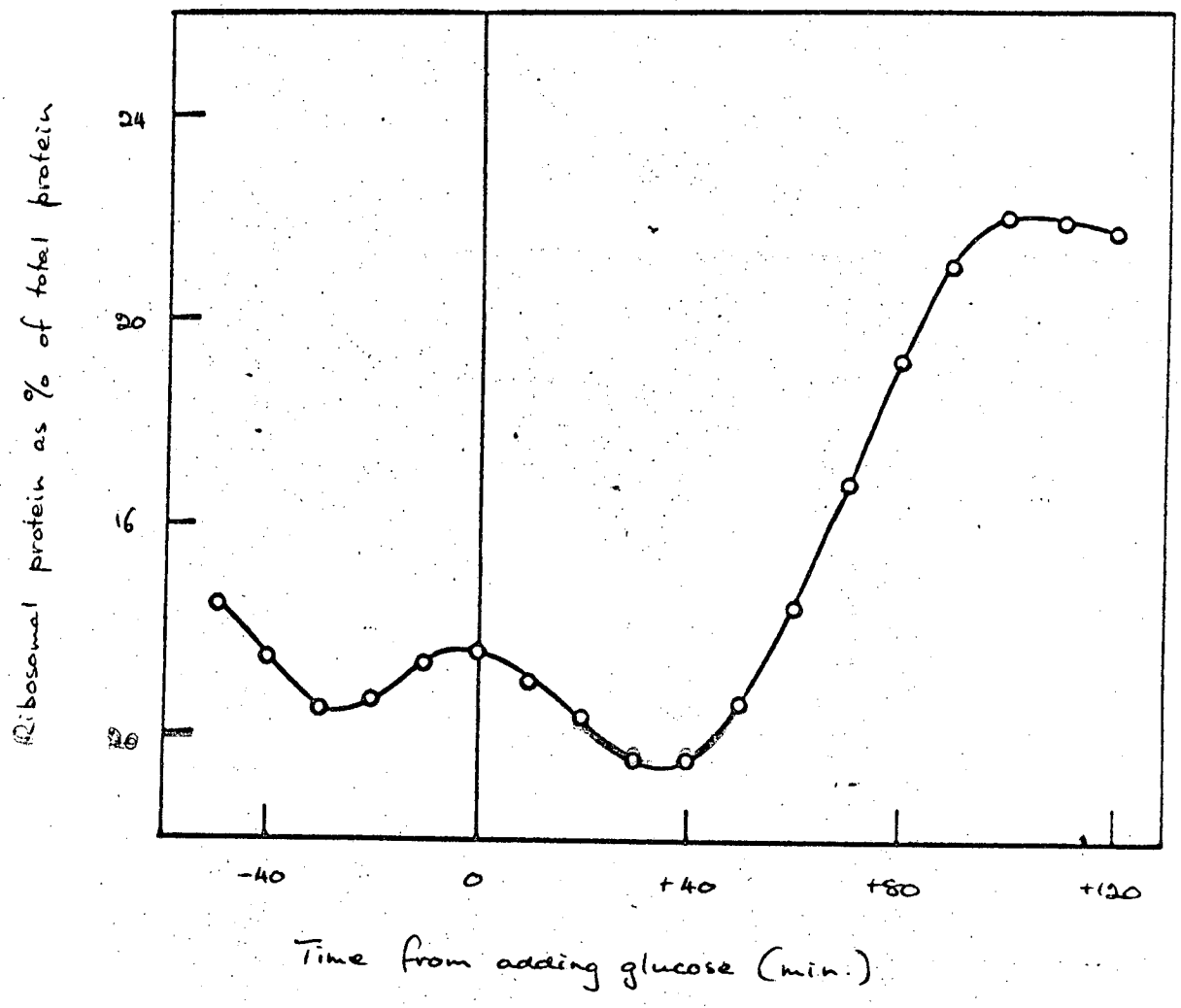
UNGAR + MOSES. FIG. 7 (TOP)



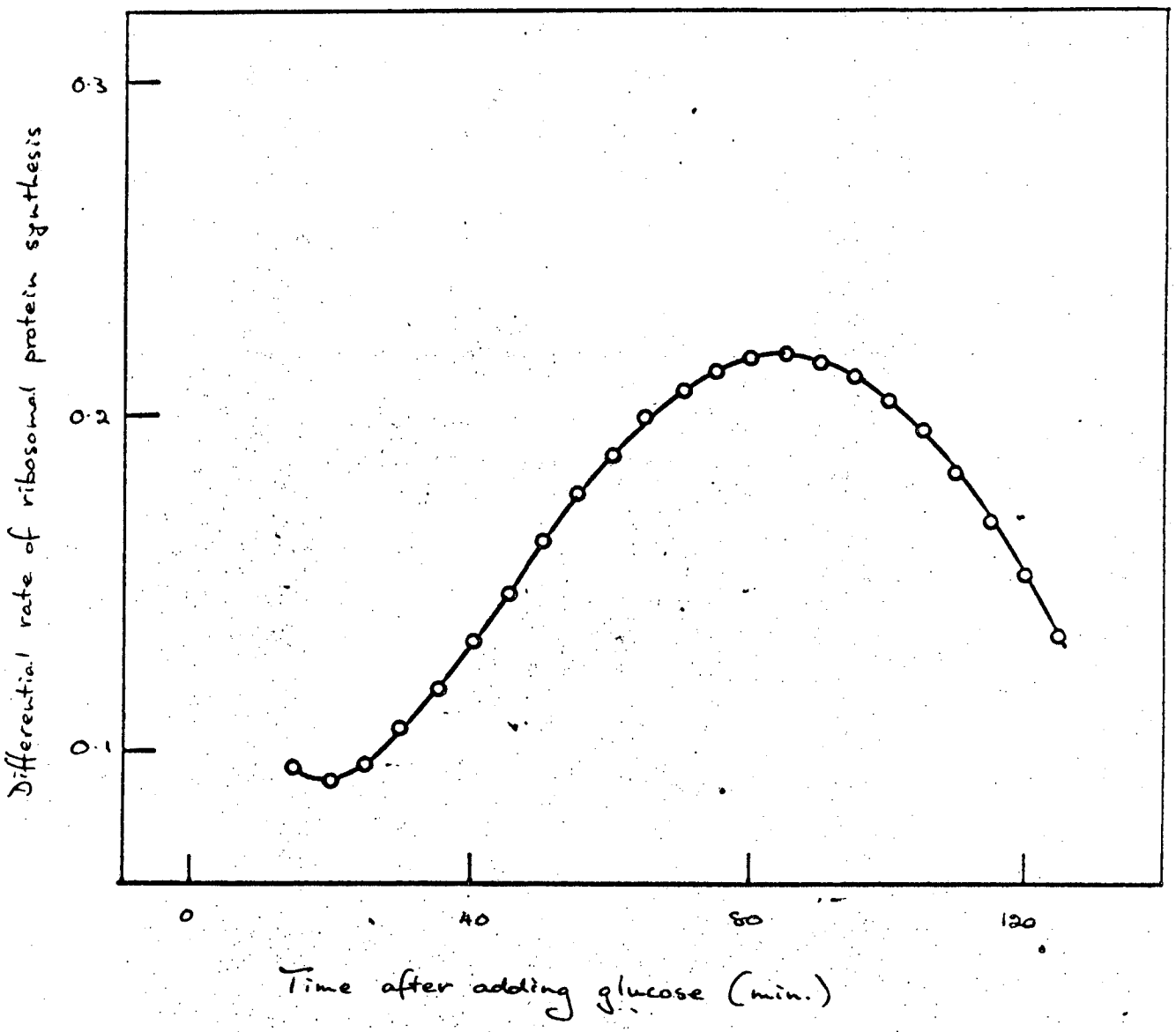
UNGAR + MOSES FIG. 7 (BOTTOM)



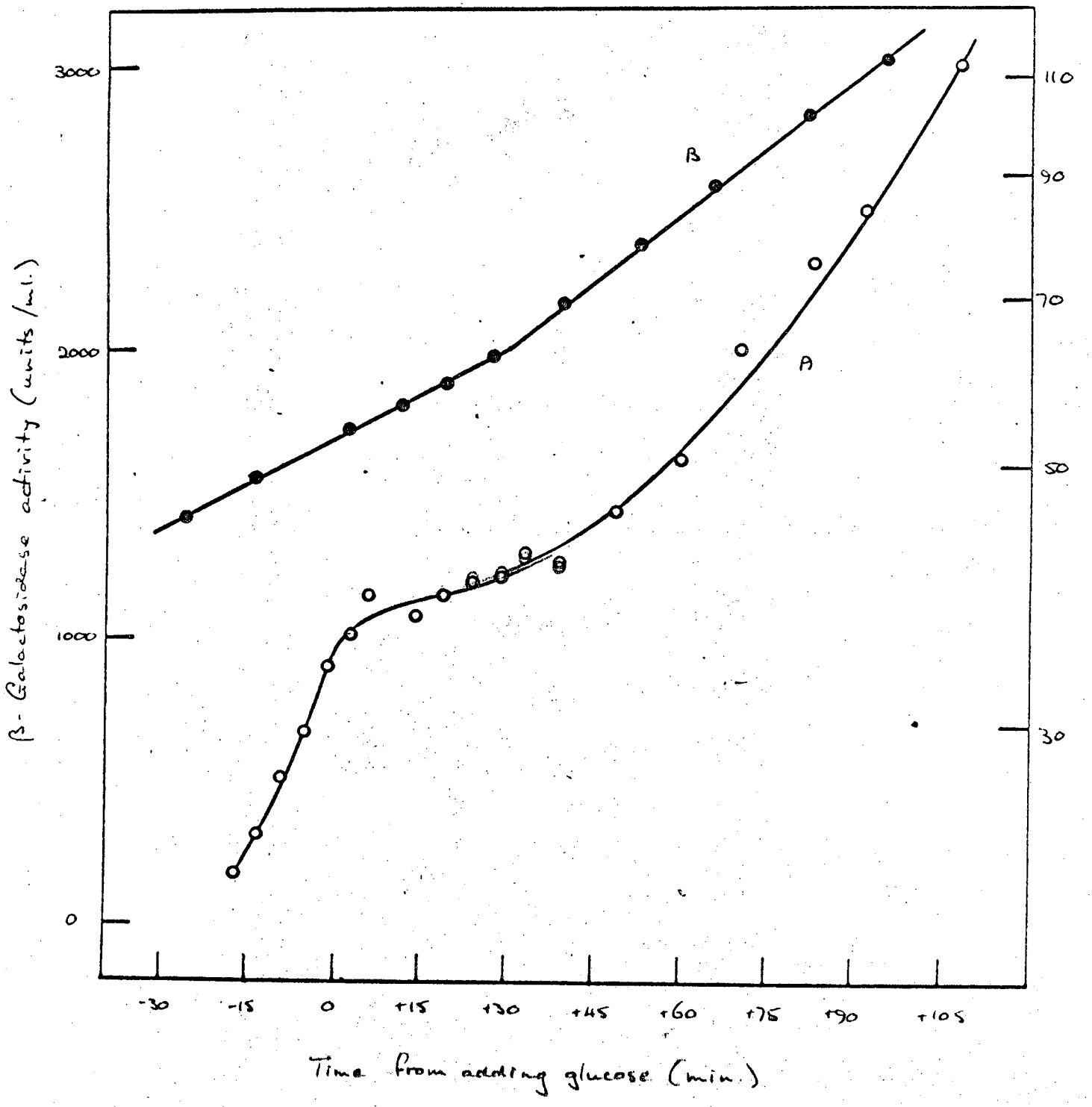
UNGAR + MOSES FIG. 8.

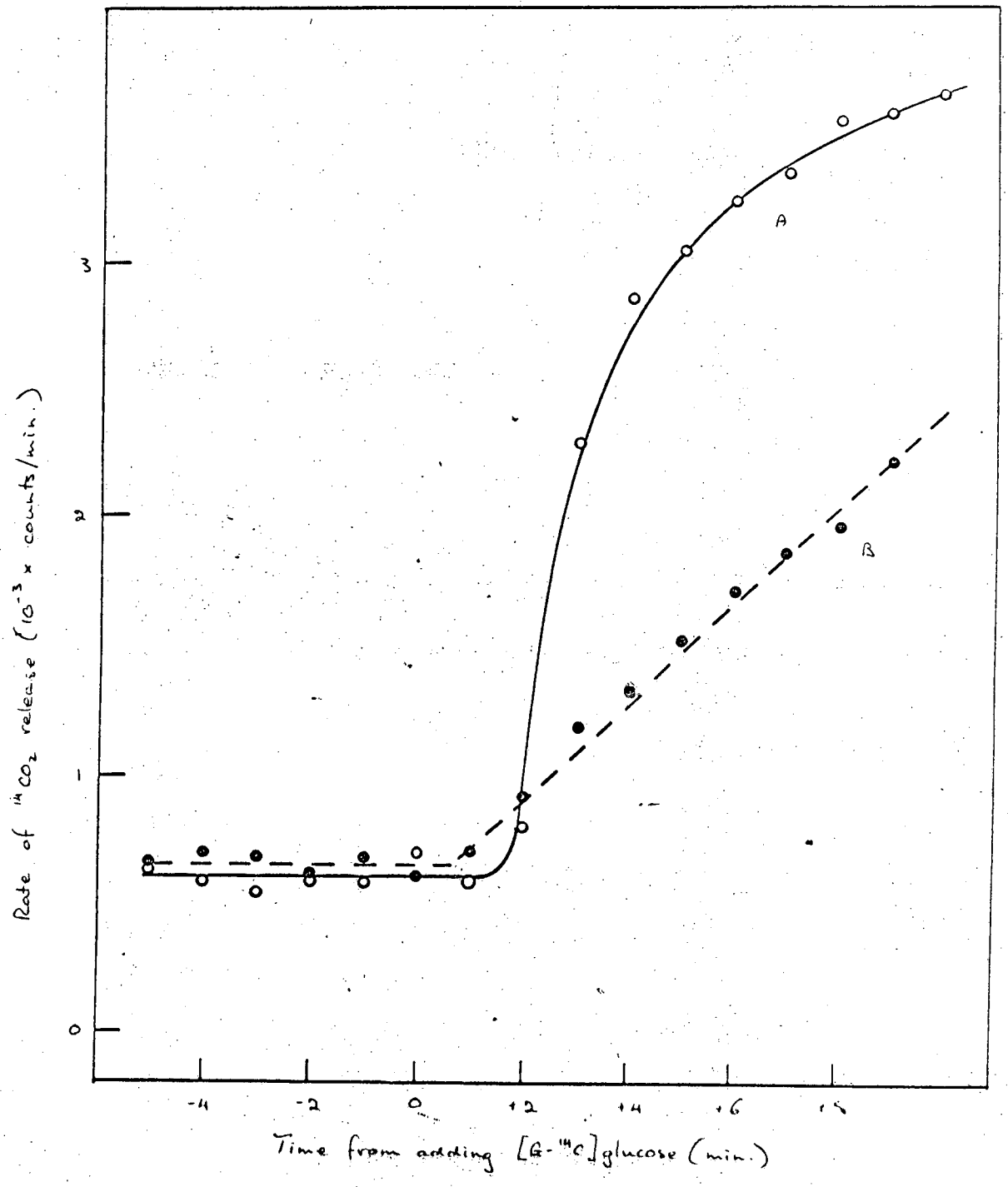


UNGAR + MOSES FIG. 9.

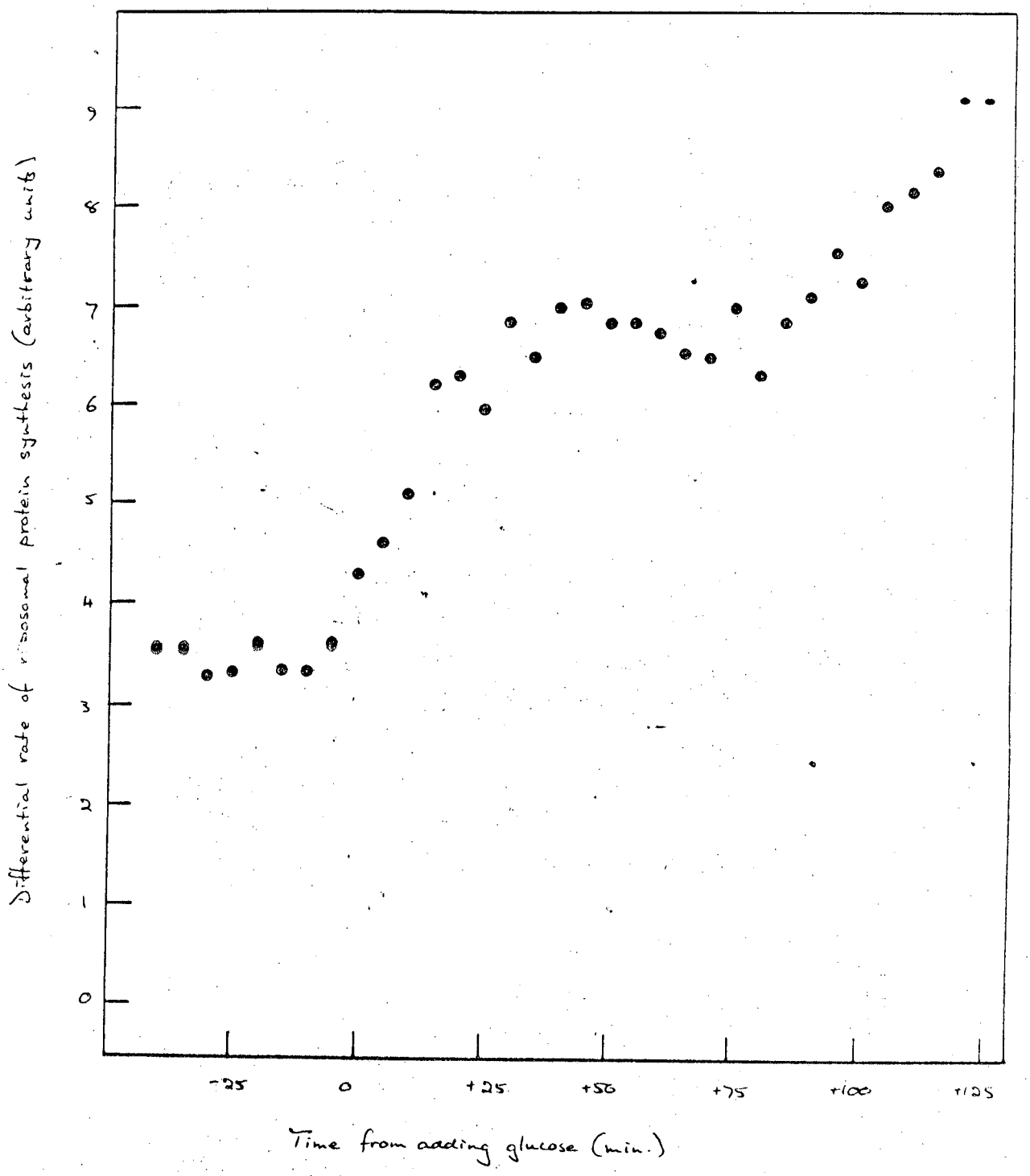


UNGAR + MOSES FIG. 10.




Rate of $^{14}CO_2$ release (10^{-3} x counts/min.)
3
2
1
0
A
B
-4
-2
0
+2
+4
+6
Time from adding [G-^{14}C]glucose (min.)

UNGAR + MOSES FIG. 12.



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