Pools and Protein Synthesis in Mammalian Cells

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From the kinetics of incorporation into protein shown by four amino acids and one amino acid analogue in suspension-cultured HeLa S-3 cells, two distinctly different patterns were observed under the same experimental conditions. An initial slow exponential incorporation followed by linear kinetics was characteristic of the two non-essential amino acids, glycine and proline, whereas the two essential amino acids studied, phenylalanine and leucine, showed linear kinetics of incorporation with no detectable delay. The analogue amino acid, *p*-fluorophenylalanine, also showed immediate linear kinetics of incorporation. There was a poor correlation between the rate of formation of acid-soluble pools and incorporation kinetics. However, the rate of formation of the freely diffusible pool of amino acids correlated more closely with incorporation kinetics. The lack of direct involvement of the acid-soluble pool in protein synthesis was also demonstrated by pre-loading of pools before treatment of cells with labelled amino acids. The results partially support the hypothesis that precursor amino acids for protein synthesis come from the external medium rather than the acid-soluble pool, but suggest that the amino acid which freely diffuses into the cell from the external medium could also be the source.

There is contradictory evidence concerning the source of precursor molecules for protein synthesis. Some claim that amino acids taken up from the extracellular medium are quickly activated and loaded on to tRNA, thereby taking a direct route to the ribosomal machinery and effectively by-passing an existing trichloroacetic acid-soluble intracellular pool of the same amino acid (Kipnis *et al.*, 1961; Rosenberg *et al.*, 1963; Berg, 1968; Hider *et al.*, 1969; van Venrooij *et al.*, 1974). Linear kinetics and an absence of delay in incorporation have been characteristically observed. Others claim that the acid-soluble pool provides the precursors, incoming molecules from the external medium having to pass into the pool and eventually equilibrating with it before linear kinetics are observed (Kemp & Sutton, 1971; Fern & Garlick, 1973; Li *et al.*, 1973; Mowbray & Last, 1974; Robinson, 1977). Incorporation therefore starts more slowly with some delay before accelerating as amino acids begin to equilibrate with the existing pool and eventually replace it. Convincing evidence has been presented in the above reports for both hypotheses. Either the two hypotheses are mutually exclusive and one is incorrect, or each has its validity within the system, or under the conditions, in which incorporation has been studied. If both hypotheses are valid in part but not in general, there must be some more unifying hypothesis providing a better explanation for the relationship between amino acid uptake and incor-

poration. For example, the present preoccupation with the acid-soluble intracellular pool might be misleading in this connection. This report presents evidence which makes this suggestion more probable and suggests an alternative. It has stemmed from an analysis of the uptake of phenylalanine and its analogue, *p*-fluorophenylalanine (*p*FPhe), in relation to their incorporation into protein, which clearly demonstrated that the conventional acid-soluble pool bears little direct relationship to incorporation (Wheatley *et al.*, 1978).

Evidence will be presented which shows that: (i) some amino acids are incorporated without delay and with linear kinetics, whereas others, in the same cell system under the same conditions, are not; (ii) pre-loading of intracellular acid-soluble pools to different extents does not affect the incorporation kinetics of subsequently administered labelled amino acid; (iii) incorporation characteristics for proline are curvilinear in both a normal and an auxotrophic mutant cell line. The results are discussed in terms of the most probable pathway of an amino acid *en route* to incorporation into protein.

Methods

Chemicals and radiochemicals

The following amino acids purchased from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.) have been employed: leucine, glycine, proline, phenylalanine and the analogue *p*-fluoro-

Abbreviation used: *p*FPhe, *p*-fluorophenylalanine.

phenylalanine (*p*FPhe). Most radioactively labelled amino acids were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. They included L-[4,5-³H]leucine at a specific radioactivity of 58 Ci/mmol, [2-³H]glycine at 23 Ci/mmol, DL-[G-³H]phenylalanine at 4.2 Ci/mmol, L-[2,4,6-³H]phenylalanine at 62 Ci/mmol and DL-*p*-fluoro[4-³H]phenylalanine at 2.0 Ci/mmol. The DL-[³H]phenylalanine product was used for a stricter comparison with the analogue, which was available only as the racemate. L-[4-³H(n)]Proline at 26.3 Ci/mmol was obtained from New England Nuclear Corp., Boston, MA, U.S.A.

Cultured cells

All amino acids were studied in HeLa S-3 suspension-cultured cells routinely maintained in exponential phase at between 1×10^5 and 3×10^5 cells/ml in basal Eagle's medium. The relevant amino acid concentrations in this medium are: 0.29 mM-leucine, 0.10 mM-glycine and phenylalanine; proline is absent. Glycine was present since the medium used was the modification by Mueller *et al.* (1962), and for this reason it also contained serine at 0.10 mM. Eagle's basal medium also contains other non-essential amino acids: cystine (0.05 mM) and glutamine (2.0 mM).

Other cell lines included CHO-10B, CHO-K1 and BHK 21/C-13/DWS-3, the last having been specifically adapted to grow in suspension culture with basal Eagle's medium to compare with HeLa S-3 cells. Landschütz ascites-tumour cells removed from 6d tumour-bearing mice were washed twice in 37°C Dulbecco's phosphate-buffered saline medium and resuspended for short-term suspension culture in Eagle's medium. All manipulations were carried out in a $37 \pm 0.5^\circ\text{C}$ hot-room.

Cells were concentrated to 1×10^6 /ml in fresh Eagle's medium 20–30 min before addition of radioactively labelled amino acid. Duplicate or quadruplicate samples of 2 ml volume (2×10^6 cells) were removed at each time point and squirted into 5 ml of ice-cold saline. The usual times taken were 1, 2, 3, 5, 10, 20, 40, 60, 80 and 100 min. Medium was sampled at the start and the finish of each experiment to check for any significant loss of radioisotope during the course of the incubation. Cell number did not change significantly during 100 min incubations.

Treatment of samples

After spinning down and washing twice in 5 ml of ice-cold saline, the pellets were processed in a similar manner to the procedure of Robinson (1977), consisting of two extractions for 30 min with ice-cold 0.2 M-perchloric acid and one extraction for 30 min at 70°C in 0.6 M-perchloric acid. Supernatants were sampled after each extraction and scintillation

counting was carried out to determine total acid-soluble pool sizes. The resulting pellets were washed twice in ice-cold ethanol, evaporated to dryness in a desiccator under vacuum conditions and dissolved in 0.5 ml of formic acid (90%). Duplicate samples were taken for scintillation counting, and further samples were used (after evaporating off the formate) for a modified Lowry analysis (Oyama & Eagle, 1956), providing an estimate of protein in $\mu\text{g}/10^6$ cells based on a bovine serum albumin (fraction V) standard. From these data, specific radioactivities, incorporated c.p.m./ μg of protein, could be calculated (but see below).

The perchloric acid-extraction technique does not give a particularly accurate picture of the intracellular pools, other than the conventional 'acid-soluble pool'. Parallel experiments or samplings were therefore run which involved a non-washing procedure before acid extraction. By means of a [¹⁴C]inulin space marker, it was possible to measure medium contamination of pellets and thereby calculate total uptake of amino acids by the cells alone. The corrected total for the uptake could then be subdivided into (i) the 'freely diffusible pool', which is washed out with saline, and (ii) the 'acid-soluble pool', extracted with perchloric acid; for details see Wheatley (1978) and Wheatley *et al.* (1978) (in which it is referred to as the 'slowly-diffusible pool' or SDP).

Scintillation

For almost all experiments, the scintillation mixture used was Triton X-114/xylene/diphenyloxazole (Fox, 1976), but on several occasions results have been checked with other scintillants less likely to give adverse reactions with perchloric acid, usually Kinard's mixture (Kinard, 1953) or Triton X-114/toluene/POPOP (Fox, 1968). Volumes were kept as small as possible to obviate some of the scintillant-perchloric acid interactions, usually not more than 100 μl being present in 10 ml of scintillation 'cocktail'. The efficiency of the Packard 2450 spectrometer was approximately 22–25% for tritium under these conditions. However, results have been expressed here without correction for efficiency because the study has been essentially of a comparative nature. In fact, data have usually been given as c.p.m./ 10^6 cells for the following reason. In most experiments of 100–120 min duration with labelled amino acids, there was a very small net increase in protein in the culture, estimated at between 2 and 4%. Not only is the Lowry test too insensitive to read such small changes reliably, its variability was found to be too great to provide accurate estimates. Thus the protein content of 80 samples each containing that of 1×10^6 cells, was $186 \pm 19 \mu\text{g}$ (mean $\pm$ s.d.). Calculation of specific radioactivities of incorporated amino acids could be made, but were not as consistent as results expressed

in c.p.m./ 10^6 cells. Furthermore, calculation of specific radioactivity of incorporation of a labelled amino acid assumes negligible conversion of the given amino acid before it enters proteins. Although there is considerable evidence to support this assumption from the earlier work of Eagle and his colleagues under experimental conditions similar to those used in the present work (see Eagle, 1959), the amount of interconversion must still be verified in our experiments before the term 'specific radioactivity' of incorporation can be strictly applied for a particular amino acid (work in progress; see the Discussion section).

Results

Pools and protein incorporation

Before describing the experimental data, certain theoretical predictions can be made of the shapes of incorporation curves which might be expected as a result of the different pathways by which amino acids are taken up and incorporated by cells, as attempted by Britten & McClure (1962). Fig. 1 depicts several of the possibilities, from rapid uptake into both pool and protein (a) to very slow entry into both (d), to which reference will be made below.

In Fig. 2(a), the results of [^3H]leucine incorporation are shown, conforming to the pattern of Fig. 1(a). This has been confirmed by a large number of similar analyses, including experiments designed to obtain more detailed analysis within the first minute of

incorporation. Quadruplicate samples have been taken in two identical experiments and standard deviations calculated at each point. Since the two lines obtained from these independent experiments fitted each other exactly, a high degree of confidence of the linearity of incorporation into protein was assured, and further statistical analysis was unnecessary to fit the best straight line (see Fig. 3). No delay in incorporation could be detected with leucine and linearity was maintained for up to 2–3 h longer than the experimental duration shown in Fig. 2. The perchloric acid-soluble pool of leucine developed quickly and reached an asymptote in about 30 min.

Fig. 2(b) shows the results of experiments with [^3H]phenylalanine which produced results almost exactly identical with those for leucine, with linear incorporation occurring for several hours and cells taking 30 min to achieve a stable acid-soluble pool. Both phenylalanine and leucine permit protein synthesis to proceed at optimal rates with the external concentrations employed here, but incorporation could be increased by 10–15% to a maximum when their external concentrations were raised 3–4-fold.

Fig. 2(c) gives the results with [^3H]glycine in experimental protocols identical with those for phenylalanine and leucine. Incorporation follows the pattern seen in Fig. 1(b), but pool formation does not occur as rapidly. The latter rises in a quasi-logarithmic fashion, but not in the exponential function expected if the amino acid from the external medium slowly equilibrated with the existing internal pool (Robinson, 1977), i.e. as depicted in Figs. 1(c) and 1(d). It continued its quasi-logarithmic increase for about 1 h and then rose in a linear manner for at least a further 2 h. Incorporation into protein followed a curvilinear pattern with an initial exponential increase changing after about 15–20 min to a strictly linear rate for several hours, i.e. linearity was achieved long before the pool had reached equilibrium.

[^3H]Proline uptake followed a similar pattern to [^3H]glycine with the acid-soluble pool continuing to rise for at least 2 h (Fig. 2d). Incorporation was also like that of glycine but with a shorter exponential phase leading to linear incorporation kinetics from approximately 10 min onwards, which was sustained for 3–4 h.

The incorporation curves in Fig. 2 do not adequately demonstrate the crucial first 10 min after addition of labelled amino acid, during which the clearest evidence of the type of incorporation kinetics can be detected. The results are therefore replotted in Fig. 3 as a composite, with each curve corrected to the same specific radioactivity of radioactively labelled amino acid, i.e. $1 \mu\text{Ci/ml}$, external medium concentration 0.1 mM. This serves to emphasize the true linearity of phenylalanine and leucine incorporation, in contrast with the early exponential nature of

Fig. 1. Theoretical curves for acid-soluble pool formation (----) and protein (—) incorporation of labelled amino acids

The curves range from both being rapid (a) to both being slow (d).

Fig. 2. Acid-soluble pool formation (●---●) and incorporation of labelled amino acid into protein (○—○) (a) [^3H]Leucine (0.29 mM, $1\ \mu\text{Ci/ml}$); (b) [^3H]phenylalanine (0.1 mM, $1\ \mu\text{Ci/ml}$); (c) [^3H]glycine (0.1 mM, $1\ \mu\text{Ci/ml}$); (d) [^3H]proline (0.3 mM, $1\ \mu\text{Ci/ml}$).

glycine and proline incorporation. The extrapolation of the leucine and phenylalanine lines to about -10s on the abscissa is consistent with the expected error in mixing, obtaining and cooling samples for the first time point, which we experienced in attempting incubation times of less than 1 min.

Similar curves to those seen in Figs. 2 and 3 have been obtained with at least three different cell lines, including CHO-10B, BHK 21/C-13/DWS-3 and Landschütz ascites-tumour cells, which suggests that the different uptake and incorporation features are not peculiar to the HeLa S-3 cell line and may apply more generally to mammalian cells. No significant difference in these patterns was detected between

Fig. 3. Replot of the incorporation results of Fig. 2 for the first five time points up to 10 min

The results are normalized to the same specific radioactivity of labelled amino acid in the external medium (0.1 mM, $1\ \mu\text{Ci/ml}$). Vertical bars give the s.d. from the mean of eight samples; these were omitted in Fig. 2 for clarity.

experiments with monolayers and suspension-cultured cell systems.

Pre-loading of pools before incorporation

One of the problems that became apparent from the above results was that of correlating acid-soluble pool size with incorporation into protein when the non-essential amino acids show continued expansion of this pool for longer than the course of the experiment. Equilibrium is reached in 30 min with phenylalanine and leucine, and predictions based on estimates of pool size and its specific radioactivity can be made. The incipient pool size of glycine and proline cannot be deduced, since they are non-essential and therefore cells presumably contain an endogenous pool of unknown size. Whether this in itself is relevant to differences in incorporation patterns will be discussed below, but for this reason subsequent analyses

on loading were first carried out with the essential amino acid phenylalanine and its analogue, *p*FPhe. This analogue is utilized at 10–20% of the efficiency of phenylalanine when competing at equimolar concentration and at 70–80% efficiency in the absence of phenylalanine. After pretreating cells with either one or the other amino acid to produce pools of different sizes, depending on the concentration of amino acid in the external medium ($[aa]_e$) employed, they were subsequently made to incorporate labelled analogue or homologue to measure interference due to the pretreatment. Thus when large acid-soluble pools of homologue had been formed, analogue molecules subsequently supplied would be expected to become incorporated with difficulty if the cells utilized the homologue acid-soluble pool as a source of precursor.

Cells were treated with 0.2–1.0 mM-phenylalanine or *p*FPhe for 1 h before treatment with tritium-labelled phenylalanine or *p*FPhe. For further

Fig. 4. Effect of pre-loading cells with phenylalanine on the subsequent incorporation of labelled phenylalanine (a) or *p*FPhe (b) (a) Incorporation of 0.1 mM- $[^3\text{H}]$ phenylalanine (1 $\mu\text{Ci}/\text{ml}$) in cells maintained in this external concentration throughout (○—○), or given this same concentration either at the same time as 0.5 mM-phenylalanine (●—●) or 1 h after 0.5 mM-phenylalanine (●—●). The absence of any difference between the last two indicates that the 1 h pretreatment at 0.5 mM (which produces a 4.2-fold larger acid-soluble pool) has failed to influence the linear uptake kinetics. ----, 0.1 mM incorporation curve for $[^3\text{H}]$ phenylalanine normalized (by one-sixth) to the specific radioactivity of the others. (b) A similar experiment to (a) but one in which 5 μCi of $[^3\text{H}]\text{pFPhe}$ has been added to cells that have received 0.2 mM-DL-phenylalanine at the same time as the analogue (■—■). The middle pair of curves are for cells that received either 0.2 mM-DL-phenylalanine 1 h before and 0.2 mM-DL-phenylalanine simultaneously with the labelled analogue (▲—▲) or 0.4 mM-DL-phenylalanine simultaneously with the analogue (▼—▼). The lower pair of curves show cells incubated with 0.5 mM-DL-phenylalanine 1 h before receiving 0.2 mM-DL-phenylalanine simultaneously (△—△), compared with 0.7 mM-DL-phenylalanine given simultaneously (▽—▽). The results prove that the preforming of pools of the highly competitive homologue, phenylalanine, fails to disturb $[^3\text{H}]\text{pFPhe}$ incorporation to any greater extent than when given simultaneously.

details of pools in this type of experimental procedure see Wheatley *et al.* (1978), but for the present purposes it need only be stated that the perchloric acid-soluble pools produced from 1 mM-phenylalanine and *p*FPhe were approximately 3.9–4.3-fold larger at equilibrium than the pools produced from 0.2 mM concentration of external amino acid. The results of two relevant experiments are shown in Fig. 4(a and b). Pretreatment of cells with different concentrations of amino acids to form pools of varying size had no detectable effect on either the amount or the linearity of incorporation of the subsequently added labelled amino acid, i.e. after normalizing the concentrations or ratios of the amino acids in the external medium. From a knowledge of the competitive behaviour of phenylalanine and *p*FPhe (see Wheatley, 1978) it was surmised that the formation of a large acid-soluble pool of phenylalanine in cells before addition of *p*FPhe would severely handicap the utilization of the labelled analogue added 1 h later. If the analogue had to pass through this pool before it became incorporated it would stand, at most, only a 1-in-5 chance of being loaded on to tRNA^{Phe} compared with phenylalanine, after it had reached equilibrium (Arnstein & Richmond, 1964; Dunn & Leach, 1967; Wheatley, 1978). The absence of a delay in incorporation and its linearity from the start is compelling evidence that the analogue can become incorporated into protein despite existing pools of 'competing' homologue amino acid, as well as external competition. These experiments are being repeated with removal of the pretreating amino acid before addition of the label. This requires monolayer work, since the shock of spinning down and resuspending suspension-cultured cells disturbs the sensitive analysis of labelled amino acid incorporation

in the early minutes after treatment. Preliminary results confirm that the outcome is similar to that reported in Fig. 4. Other pre-loading experiments with leucine and phenylalanine have also failed to influence the linear kinetics of their incorporation. Particularly good verification on this point was obtained when we returned to an examination of the non-essential amino acid, glycine. Cells were preincubated for 1 h in this amino acid at 0.1 and 1 mM before being given a change of medium to 0.1 mM (1 μ Ci/ml). The pretreatment in this series of loading experiments failed to influence the shape of the incorporation curve, and therefore gave results like those seen in Figs. 2(c) and 3, regardless of the acid-soluble pool sizes being grossly different before the label was added (the pool at 1 mM was 5.6 times larger than at 0.1 mM).

Acid-soluble versus free amino acid pools

Since previous work had shown that the acid-soluble pool forms independently of protein synthesis and only becomes related to [aa]_e for amino acids such as leucine and phenylalanine at equilibrium (van Venrooij *et al.*, 1974; Wheatley *et al.*, 1978), it was not unexpected to find a poor correlation within the initial 30 min between incorporation and this pool size. The problem of the differences in uptake, shown in Fig. 2(a–d), awaits a detailed analysis of the correlation between incorporation and different intracellular pools for many more amino acids and from different external amino acid concentrations (work in progress). But observations with [³H]-glycine and [³H]proline, based on the same non-washing type of analysis which has proved informative for phenylalanine (Wheatley *et al.*, 1978), suggest

Fig. 5. Uptake curves of freely diffusible pools in HeLa cells from 0.1 mM (1 μ Ci/ml) external medium concentration of amino acids
(a) [³H]Leucine (■) and [³H]phenylalanine (●); (b) [³H]glycine (□) and [³H]proline (▲).

Table 1. Ratio of freely diffusible to acid-soluble pool of amino acids in HeLa cells over the first 2h after treatment

Values for phenylalanine give the ranges of ratios found in four separate experiments. Each ratio is taken as the accumulated radioactivity in the 'freely diffusible pool' divided by the corresponding amount in the 'slowly diffusible pool' (or, more conventionally, the acid-soluble pool) at the stated time points. The changing of the ratio with time for each of the amino acids is the important point; comparisons made laterally should not be construed as accurate indications of differences in the absolute sizes of pools between the amino acids.

Time (min)	Phenylalanine	Glycine	Proline
2	21-22	—	—
5	6.5-7.1	2.22	2.08
10	5.1-6.0	2.17	1.38
30	3.2-4.6	1.67	0.76
60	3.0-4.1	1.78	0.62
120	3.3-3.8	2.18	1.05

that the rate during the initial stages of incubation is largely governed by the speed with which the amino acid enters the cell by free diffusion. At 4 and 37°C, the freely-diffusible pools of leucine and phenylalanine achieve equilibrium virtually instantaneously. This pool also represents about 70-75% of the total amino acid uptake once equilibrium has been reached at a physiological [aa]_c, the acid-soluble pool accounting for the rest. Initially, however, there is a huge preponderance of the free amino acid because the acid-soluble pool takes 30min to reach equilibrium. A similar analysis for glycine and proline showed that they differed from the essential amino acids in forming 'freely diffusible' pools much more slowly (Fig. 5). Thus the ratio of the two pools was both closer and more nearly constant from the start than those for phenylalanine and leucine (Table 1).

Incorporation in the auxotrophic mutant cell line CHO-K1

Finally, the problem of whether dynamics of incorporation are in some way dictated by the cellular requirements for an amino acid, i.e. whether it is essential or not, was tested by comparing two sub-lines of the Chinese hamster ovary cell line originated by Tijo & Puck (1958). One, designated CHO-10B cells and adapted to Eagle's medium, grows in the absence of proline, whereas the CHO-K1 cells have a requirement for this amino acid. Both cell lines were grown in basal Eagle's medium containing proline until 2h before analysis, when each of the cultures was divided into two, one half getting no proline and the other having 0.3mM-proline added to the medium (which had dialysed serum present). At the start of the labelling period 2h later, all cultures were changed to medium containing 0.3mM-

Fig. 6. Uptake of [³H]proline by CHO-10B cells (□---□) and by the proline-requiring mutant CHO-K1 cells (■---■)

After resuspension, cells were treated with proline at 0.3mM (3μCi/ml). The K1 cells were proliferating at about 70% of the rate of the CHO-10B cells during the 16h period preceding the addition of labelled proline.

proline with [³H]proline present (3μCi/ml). There were no differences between the two groups or the two cell lines with regard to the patterns of uptake and incorporation, i.e. the auxotrophic mutant showed the same slow exponential incorporation over the first 10-20min before linear kinetics were established.

This experiment was repeated in short-term suspension-cultured CHO-10B and CHO-K1 cells. In this case, cells were deprived of proline for 2h before it was restored to the culture medium as before at 0.3mM (3μCi/ml). As in monolayer studies, the slower growing K1 strain of the CHO stock incorporated only 70-75% as much proline as the 10B line, which has no dependency upon it. After allowing for this overall slower rate of utilization by the K1 cells, both cell lines showed similar incorporation kinetics (Fig. 6). Studies on uptake of radioactivity into the 'freely diffusible' pool suggest that both cell lines accumulate proline slowly, which probably accounts for the retarded incorporation with its curvilinear pattern, as described in Figs. 2 and 3 for HeLa S-3 cells. (Incorporation of [³H]leucine followed linear kinetics in both sub-lines, and pool formation compared closely with the kinetics for HeLa S-3 cells.)

Discussion

The fact that there are at least two different patterns of uptake and incorporation of amino acids questions

any general hypothesis concerning the precursor supply for protein synthesis which is based on results obtained with a single amino acid (Adamson *et al.*, 1972; Fern & Garlick, 1973; van Venrooij *et al.*, 1974; Hod & Herskho, 1976). Either there are multiple mechanisms and no generally applicable hypothesis, or the common denominator which underlies these differences has not been found. The problem is further complicated by the fact that, although the kinetic behaviour seems reasonably consistent for any particular amino acid in three mammalian cell types, the same kinetic behaviour will probably not apply for other biological systems such as sea-urchin eggs (Berg, 1968) or bacteria (Britten & McClure, 1962).

The present results make it highly improbable that the acid-soluble intracellular pool, under normal physiological conditions, acts as a direct precursor pool, which is contrary to the conclusions of others, including Fern & Garlick (1973), Mowbray & Last (1974) and Robinson (1977). The linear incorporation kinetics for phenylalanine and leucine (Figs. 2 and 3) cannot be explained by amino acid passing through the acid-soluble pool unless it is infinitely small, which is not the case. The pre-loading experiments (Fig. 4) also preclude all possibility of direct acid-soluble pool involvement. Further evidence that substantiates this latter contention is to be found elsewhere (Wheatley *et al.*, 1978). Not only is the passage of essential amino acids through the acid-soluble pool improbable, the incorporation kinetics of glycine and proline would also be difficult to explain on this hypothesis, since (i) these amino acids become incorporated at linear rates long before their acid-soluble pools have established equilibria with the external medium, (ii) their uptake kinetics into the acid-soluble pool are nevertheless logarithmic and not sufficiently different from that of phenylalanine and leucine up to 30 min to account for their slow exponential incorporation into protein, and (iii) pre-loading experiments also demonstrate that it is highly improbable that the acid-soluble pool directly supplies protein synthesis.

The results reported here agree with those of Robinson (1977) as far as uptake and incorporation kinetics of proline and glycine are concerned. His results with leucine incorporation are contrary to our findings. We have observed linear incorporation without delay in a large number of experiments; our suspension culture system lends itself particularly well to this type of study because manipulations are reduced to the minimum. Large cultures can be treated instantly and mixed within seconds by the orbital shaking. The same cultures are sampled subsequently and therefore all time points relate to the same original population of cells, which obviates variations met when groups of monolayer cultures are used. Our findings also disagree on the utilization of the

acid-soluble pool. Calculations were made on the four amino acids used in this study to assess how long protein synthesis could be sustained if all the acid-soluble pool was discharged into protein after cells had been incubated at 0.1 mM ($1 \mu\text{Ci/ml}$) for 1 h and then transferred to unlabelled medium. The estimates were 12 min for phenylalanine, 13 min for leucine, 98–136 min for proline and 158–190 min for glycine. Experimental analyses to establish what in fact happens to this pool are in progress, but even allowing for a loss of about 2% of existing protein label through degradation in the chase period (see Wheatley *et al.*, 1977), no evidence has been found of a significant movement of labelled amino acid from the acid-soluble pool into protein in any of the four cases.

Evidence for precursor hypotheses that are based on glycine and proline should probably be viewed cautiously, not only because they are both non-essential amino acids for many eukaryotic cells (see below), but because results of an analysis of a whole spectrum of amino acids, including other non-essential ones (D. N. Wheatley & J. H. Robertson, unpublished work) show that they are exceptional and not typical in their kinetic behaviour. They are both highly dependent upon Na^+ for transport, and more work will be necessary at 4 and 37°C on the formation of their various intracellular pools to analyse what is responsible for their different behaviour. The acid-soluble pools they produce also appear to be greatly expandable in contrast with others (Figs. 2c and 2d), which explains how they could potentially support protein synthesis for much longer than phenylalanine and leucine pools in the above estimates. Even assuming that the correlation of acid-soluble pools with protein synthesis was valid and meaningful, the fact that cells can produce these amino acids endogenously makes analysis far more complex than with essential amino acids. Their non-essential nature should not be considered responsible, as such, for slower incorporation. In addition to the reasons already discussed, there is the observation that the auxotrophic mutant CHO-K1 cells show similar uptake kinetics to CHO-10B cells. Although this negative evidence cannot allow firmer conclusions to be drawn, it nevertheless illustrates that the presence (or absence) of an endogenous supply or pool of an amino acid (proline) probably plays no regulatory function in uptake kinetics.

The role of the endogenous synthesis, and its effect upon incorporation characteristics of exogenous labelled amino acids, will be difficult to assess. Although for proline the results suggest that endogenous synthesis plays no significant part, the situation with glycine is much more complex. It is converted into many other metabolites and therefore incorporation need not necessarily be as glycine, nor indeed only into protein. It is probably not meaning-

ful to pursue the question of its incorporation kinetics further without undertaking a full investigation into the nature of the labelled products in protein and these experiments are in progress. The metabolic interconversion of these amino acids may give rise to spurious estimates of their incorporation into proteins on a specific-radioactivity basis, although there is considerable evidence that under similar experimental conditions to those employed in this report, HeLa cells do not convert these amino acids into others to a significant extent, including glycine into serine (Eagle, 1959). Surprisingly, glycine and proline (the latter to a lesser extent) have nevertheless been frequently chosen for incorporation studies (Kipnis *et al.*, 1961; Rosenberg *et al.*, 1963; Adamson *et al.*, 1972; Fern & Garlick, 1973).

Since the evidence is largely contrary to the claim that the acid-soluble pools are directly involved in precursor supply for protein synthesis, an alternative explanation is the possibility that the 'freely diffusible pool' is involved. This can adequately explain both the absence of delay and linear kinetics of incorporation of the essential amino acids, and the slower incorporation of proline and glycine (see Fig. 5). Little attention has been paid to the large scale, and usually very rapid (within seconds), influx and efflux of amino acids across the cell membrane. In most conventional studies, cells that have been labelled with amino acids are washed several times in saline at 0°C to remove extraneous radioactivity counts. This procedure washes free amino acid out of cells as it washes contaminating (extraneous medium) radioactivity off. When this is done thoroughly, as has been carefully described by van Venrooij *et al.* (1974) and Hod & Hershko (1976), only the acid-soluble pool remains. It is not surprising, therefore, that investigators have been preoccupied with this pool in relation to protein synthesis. Some, e.g. Kipnis *et al.* (1961), have recognized the importance of free amino acid pools, but their use of whole-tissue or organ preparations for analyses of this kind makes matters far more complex. The freely diffusible amino acid pool in cells can also provide an adequate explanation for the pre-loading experiments. Irrespective of the intracellular concentration, resuspension or treatment of cells at another extracellular concentration will rapidly (within seconds) result in the equilibration across the membrane. The rate of incorporation will thus be largely dictated by the external concentration resetting the internal free amino acid concentration, and not by the loading of other cellular pools.

The tentative conclusion is reached that the uptake and incorporation kinetics of several amino acids can be more easily related to the behaviour of the truly free amino acid pool inside cells than to the acid-soluble pool. Most amino acids equilibrate virtually instantaneously across the cell membrane and there-

Scheme 1. Diagrammatic representation of the flow of amino acid into cells

The diagram accounts for immediate uptake at a rate dependent on freely diffusible pool (FDP) formation and also for the formation of slowly diffusible pool (SDP; acid-soluble pool) as an independent function from protein incorporation. If a membrane site is responsible for activating and loading amino acids on tRNA, then the left-hand dashed line would apply (---○---), and a separate pathway (---□---) would be responsible for SDP production. This Scheme has not incorporated the features of amino acid recycling or the loss of amino acids from the cell after proteolytic degradation, which would be inappropriate to include at this stage of the analysis. $[aa]_0$, Concentration of amino acid in the external medium.

fore the intracellular specific radioactivity of the freely diffusible pool immediately reflects that of the external concentration. Protein synthesis would proceed at a rate commensurate with the speed at which equilibrium of the free amino acid is established, i.e. virtually instantaneous for leucine and phenylalanine but slower for glycine and proline. The reason for the slower diffusion or retention of glycine and proline across the cell membrane is obviously an important problem, which requires elucidation. The acid-soluble pools form simultaneously with the influx of amino acid, but always take time to reach equilibrium and are also dependent on metabolic energy. Although they develop as protein synthesis proceeds, it is clear that the two processes are independent and not causally related (Scheme 1).

The ultimate precursor supply for protein synthesis is the aminoacylated tRNA molecules. In the case of leucine this pool equilibrates with extreme rapidity

on addition of labelled amino acid (van Venrooij *et al.*, 1974). We are now left with two major possibilities concerning the uptake of exogenously supplied amino acid for protein synthesis: (i) the freely diffusible pool is used with an intracellular enzyme complex activating and loading the free amino acid on to tRNA, or (ii) the external medium is the source, and the activating-load enzyme complex resides in the plasma membrane. The membrane enzyme complexes would be highly polarized and act in a concerted manner to load tRNA directly, as suggested by Loftfield & Eigner (1969). In the first case, the rate of diffusion of amino acids across the membrane would be rate-limiting, whereas in the second case, different affinities of the enzyme systems in the membrane would have to be postulated to explain the differences in the availability of amino acids such as glycine and proline for protein synthesis. The fact that the aminoacyl-tRNA synthetases are not exclusively localized in membrane fractions (Neidhardt *et al.*, 1975) does not help to decide between these alternatives, but other experimental approaches might be capable of testing which pathway is involved. The simple diagrammatic representation of the situation (Scheme 1) illustrates the possibilities, with the dashed arrow representing a possible selective (enzymic) uptake-activating-loading mechanism at the membrane. A link between the acid-soluble pool and protein synthesis has not been ruled out, but so far we have not found any firm evidence for it (D. N. Wheatley & J. H. Robertson, unpublished work). Under certain physiological conditions it may assume importance, but this is entirely speculative. Adamson *et al.* (1972) have suggested that the acid-soluble pool can be indirectly involved but only by returning amino acids to the membrane for reprocessing.

In this discussion we have attempted to deal with the relationship of pools and proteins that form in the presence of exogenously labelled amino acid. So far we have not discussed the problem of reutilization and how this affects the results, because this may confuse rather than clarify the issues on the afferent aspects (i.e. initial rates of protein anabolism). If turnover is as extensive as some suggest, e.g. Righetti *et al.* (1971), certain corrections will have to be made to the kinetic data, especially with regard to the linearity of protein incorporation after the initial few minutes of incubation, since it must in fact only approximate to linearity. Nevertheless, it seems from our observations that the release of incorporated amino acids would result in their reutilization with a higher degree of efficiency than could be accounted for by the return of these molecules through the acid-soluble pool, especially under 'chase' conditions where a high concentration of unlabelled amino acid is present (Wheatley *et al.*, 1977). For this reason it is

probably easier to consider that a certain percentage turnover does occur for each amino acid, without this seriously disturbing the end point of our present observations, i.e. the net accumulation of exogenous amino acid in protein with time.

A perplexing question which remains is the significance of the acid-soluble pool within cells. Is it a repository of potential precursor produced as long as amino acid is available, but not put into direct use; or is it a store of amino acid converted into some chemical form for purposes unrelated to protein synthesis? Two immediate problems will be to follow the specific fate of acid-soluble pool molecules more closely and to identify the true chemical nature of its constituents. There is now increasingly persuasive evidence that this is not in fact a simple pool but that it can be subdivided into different compartments. Ward & Mortimore (1978) have already distinguished a compartment that does not equilibrate with [aa]_e as the catabolic pool of acid-soluble amino acids from an equilibrating pool associated with a 10000g pellet of rat liver homogenized in 0.25M-sucrose.

With regard to the other cellular systems, Britten & McClure (1962) questioned whether the acid-soluble pool represents a 'side-line' or a 'main-line' in bacterial protein synthesis. Their analogy succinctly summarizes the problem. In mammalian cells, our results would strongly favour the 'side-line', whereas Britten & McClure (1962) came to the opposite conclusion with *Escherichia coli*. It is interesting to find that they used proline in their studies, but the objection raised to use of this amino acid would probably not apply to a prokaryotic system. In yeast, evidence has already been presented that the acid-soluble pool does not correlate with protein synthesis (e.g. Stebbins, 1972).

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