concerned, at least 2 primitive coronary arteries are detected, especially on the right side in all the cases observed. These primitive coronary arteries decrease in number in the later stages, when they seem to fuse side-to-side to form a single coronary artery, because the remaining shallow pits are observed on the casts at the next stage (fig. 3). However, the fusing process has not been clarified, and is now under investigation. These phenomena in the development of coronary arteries should be of much help in understanding the variations of adult coronary arteries[12]. Contrary to some researchers' descriptions[3,9], our research showed that the sprouting of the left primitive coronary arteries is not earlier than the right. Figure 5 shows a general TEM view indicating one of the primitive right coronary arteries observed at stage 31. The aortic sinus wall is a multilayered structure (7-10 layers) of alternating primitive vascular cells and intercellular layers. The surface of polymorphic endothelial cells displays occasional microvilli. Further, a narrow vascular lumen communicating with the aortic lumen is observed in the sinus wall. The structure corresponds to the primitive coronary artery observed on the cast at stage 31. The primitive endothelial cells covering the arterial lumen take irregular forms and are arranged vertically to the sinus wall. Some of these are presumably degenerating cells as suggested by their electron opacity. Figure 6, obtained from the embryo at late stage 30, is very suggestive with respect to the process of formation of the primitive coronary arteries. The large pale cells, surrounded by irregularly shaped electron opaque cells containing various sizes of vacuoles, are localized vertically to the aortic luminar surface, and as a result intersect the circularly arranged cell layers. It cannot be decided definitely whether the pale cells and dark ones are derived from the aortic endothelium, since their cellular characteristics differ from the endothelial cells observed on the aortic surface. It seems likely that the canalization of the primordium follows cell death. As a result, canalized primordia fuse with the pre-existing capillaries in an end-to-end or end-to-side fashion to form the primitive coronary arteries. It remains to be shown whether or not the pre-existing capillaries inductively participate in the formation of the primordium of the primitive coronary arteries.

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On the problem of linear incorporation of amino acids into cell protein

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Summary. Incorporation of amino acids into mammalian cell protein shows immediate linear kinetics when measured at intervals as small as 5 sec. Free amino acids equilibrate instantaneously across the cell membrane, acting as the precursor supply. Glycine, which does not initially show linear kinetics, may have a significant endogenous precursor supply, but this is not the acid-soluble pool.

A simple but unexplained observation is that amino acids are incorporated into protein in cultured mammalian cells with linear kinetics[2-4]. Culture conditions facilitate studies on initial rate of incorporation because cells are bathed in a plentiful supply of medium, unlike the in vivo situation where extracellular fluids equilibrate less rapidly. No part of a monolayer cell is more than about 1 µm from the medium (or about 7 µm for a suspension cultured cell), allowing instantaneous equilibration of free amino acid molecules across the cell membrane[7-9]. It is noteworthy that other free-living organisms such as *E. coli*[5] and *Tetrahymena*[6] show linear incorporation kinetics into protein.

Where delays in incorporation occur, these are often related to the size of the acid-soluble pool and the speed with which incoming amino acids equilibrate with it. Substantial evidence[10,11] now shows that this pool does not play a precursor role in protein synthesis. Apparently contradictory evidence of Robinson[12], after correction of technical short-comings, actually corroborates that the pool is not the precursor supply[11]. The controversy is kept alive by the suggestion[13,14] that only a small part of the pool, i.e. a rapidly equilibrating 'membrane pool', provides precursors. Since the intracellular acid-soluble pool of phenylalanine or leucine contains sufficient molecules to sustain protein synthesis for only 5 min[8], the question is raised of how strict is the initial linearity of incorporation of amino acids into protein? This paper demonstrates that a) linear kinetics occur immediately after the addition of a labeled amino acid, and b) the acid-soluble pool is not directly involved, even in the case of glycine where delay occurs before linear kinetics are established[7]. Experiments reported here involved HeLa S-3, 3T3 and BHK21/C13 cell cultures.

Results. 1. *Preloading of cells.* The acid-soluble pool of an amino acid expands with increasing external concentration[14,15]. By preloading cells, any precursor component of the pool would be more evident because a) incorporation rates rise with availability of exogenous amino acids[10,14] and b) an intracellular or 'membrane' pool would increase to maintain its equilibrium with the medium. Cells were preloaded with 0.1, 1.0 and 10×10^{-3} M leucine in basal Eagle's medium + 10% neonatal calf serum for 30 min, spun out and resuspended in medium with 10^{-4} M leucine

+ ^{3}H-L-leucine at 1 μCi/ml (1 μCi/10^{-7} moles) (full details by Wheatley and Robertson[10] and legend to fig. 1).

Figure 1a shows that strict linearity occurs irrespective of the level of preloading. The preloaded pool at 10^{-3} M and 10^{-2} M were 7 and 35 times more radioactive than at 10^{-4} M respectively[10]. Incubating unpreloaded cells in labeling medium removed from these cultures gave similar data (fig. 1b), showing that the differences in slopes are due to the fall in specific activity of the labeled leucine as preloaded pools are discharged[11]. The absence of detectable delays suggests that if a small precursor pool exists, early incorporation must be analyzed in sec rather than min.

2. *Early incorporation kinetics.* Kinetics of incorporation were measured at 5-sec intervals using an adapted Zipette dispenser apparatus (Jencons Ltd, Hemel Hempstead, England) in which equal volumes of cell suspension and labeling medium were allowed to mix precipitously before rapid sampling by pumping. Data for uptake into the acid-soluble pool[16] and into protein were obtained with ^{3}H-L-leucine in medium containing 10^{-4} M leucine (as above). Figure 2 confirms that a) incorporation is linear from the start and b) equilibrium of the acid-extractable pool takes about 10-15 sec. Since the former line goes through the origin, any pre-existing precursor pool could not have sustained synthesis for a second. This also applies for phenylalanine, valine, and arginine. The data suggests a) immediate equilibration of free amino acids occurs across the membrane, b) these molecules are the precursors used in protein synthesis[17,18], and c), by inference, the acid-soluble pool does not contain free amino acid molecules[20]. Rapid exchanges of free molecules across the plasma membrane can occur at 2 °C and displace the pool[19], indicating that energy is not required for uptake.

3. *Kinetics of glycine incorporation.* Glycine and proline show delay before linear incorporation kinetics are established[7,10], but the reason for this has not been found. It is difficult to avoid implicating a substantial intracellular precursor pool equilibrating with exogenous ^{3}H-glycine[7]. Cells were incubated in medium with glycine present at 5×10^{-5} M to 5×10^{-3} M and a constant sp. act. of ^{3}H-glycine of 1 μCi/10^{-7} moles. The effects on the delay before linear incorporation occurred (fig. 3), show that exogenous glycine reduces the delay in a complex inverse relationship to a function of its exogenous concentration. One interpretation is that ^{3}H-glycine enters an existing glycine 'pool' from which residues are taken randomly for assimilation[5], but a non-equilibrating component is also present. Furthermore, this glycine precursor 'pool' is unlikely to be the acid-soluble pool[7,10,11].

Whatever constitutes the precursor pool, results with most amino acid suggest it is too rapidly exhausted (< 1 sec) to disturb linear kinetics, whereas glycine and proline pools are appreciable. Glycine shows a much slower rate of flux and pool displacement than other amino acids[16,18,19]. However, other nonessential amino acids do not conform with this behavior pattern. Serine, for example, shows similar uptake kinetics to glycine, but strictly linear incorporation into protein. Alanine is rapidly taken up and also shows linear incorporation into protein. Uptake into the acid-soluble pool offers no guide to incorporation kinetics, as expected of a pool on the efflux side of selection for protein synthesis[17] (fig. 4).

Discussion. Figure 4 is a diagram of the pathway of amino acids through the cell to help explain the data. To become incorporated, a substance must first be identified as an amino acid, and second as a specific type of amino acid for selection by an appropriate aminoacyl-tRNA synthetase. The precursor supply is truly free amino acid in equilibrium with the medium, and activation (step 2, fig. 4) is a random process. The pathway allows immediate presenta-

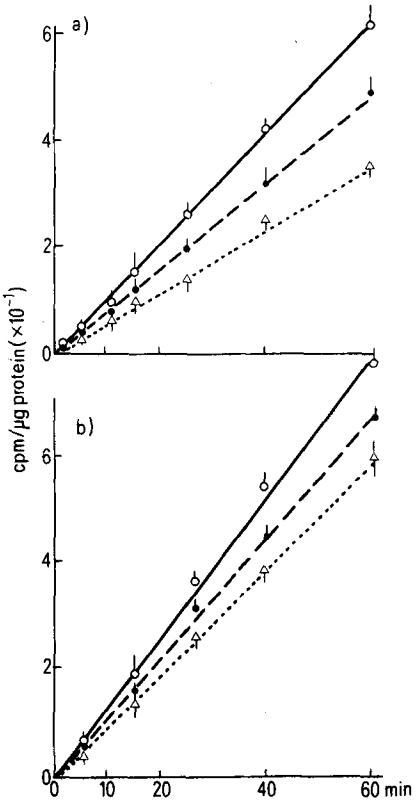

Figure 1. Incorporation of ^{3}H-leucine into cells preloaded with unlabeled leucine at 10^{-2} (△), 10^{-3} (●) or 10^{-4} M (○). Each point is the average of 6 values, bar equals 1 SD. Samples of 2×10^6 cells were spun 3 times ice-cold, 0.15 M saline. Acid-soluble pools were removed by 2 washes of 0.5 ml 0.6 M perchloric acid, the latter at 60 °C for 30 min to remove amino acid bound to tRNA[20]. Pellets were washed 2 times in saline at pH 9.5 and dissolved in 1.0 ml 1 N NaOH. 200-μl duplicates were placed in 4 ml 'Supersolve' (Koch-Light, Colnbrook, Bucks, England) and counted in minivials at 29-30% efficiency. Regression lines fitted by the method of least squares. *a* Results with cells preloaded before treatment with ^{3}H-leucine. *b* Results with fresh cells added to medium obtained at 60 min from cultures in a (see text).

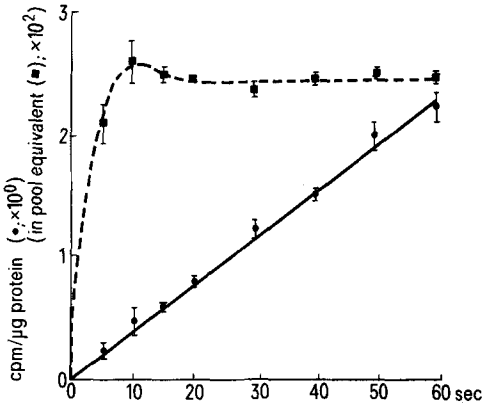

Figure 2. Incorporation into protein (●) and uptake into acid-extractable pool (■) of leucine in cultures of HeLa cells. Combined data of 2 separate experiments (running for 40 min) giving virtually indistinguishable results. Slope for incorporation fitted as described in figure 1.

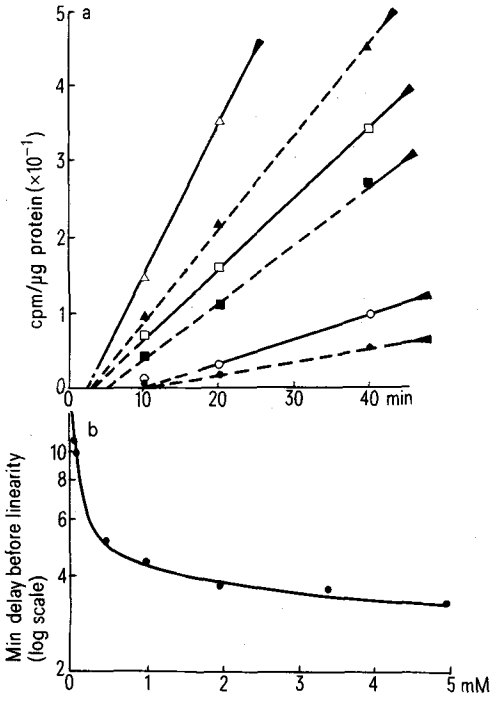

Figure 3. *a* Incorporation for ^{3}H-glycine, available at constant specific activity in the medium with final concentration of 5×10^{-5} M (●), 10^{-5} M (○), 5×10^{-4} M (■), 10^{-4} M (□), 5×10^{-3} M (▲) and 10^{-3} M (△). Data within the first 10 min has not been plotted in order that the calculated best-fit lines extrapolated back from 100 min (▲) can be clearly seen at their intersection with the abscissa. *b* Semi-log plot of time to reach linearity against glycine level. Data shows at least 3-min supply of glycine exists which is not displaced by exogenous molecules.

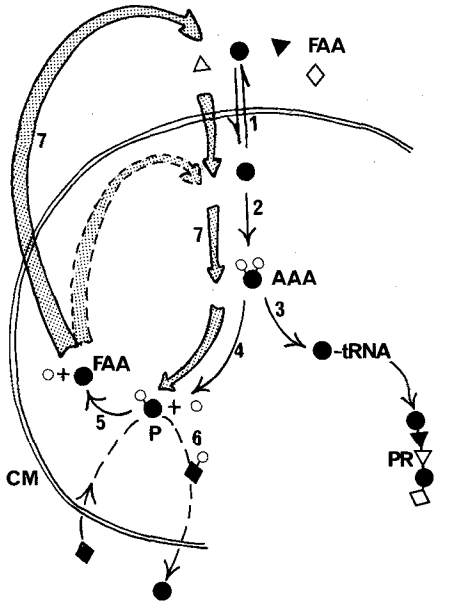

Figure 4. Model, based on Wheatley and Inglis[19], demonstrating flux of amino acid through cell to explain linear incorporation. FAA (free amino acids) in equilibrium across the membrane (CM) are the precursors (1 → 2) which can be activated (AAA). At this stage, pathway 3 or 4 occurs. In the former, capture of the AAA by appropriate tRNA-synthetases, loading on to tRNA and incorporation into protein (PR) ensues. Most AAA, however, decays to a less activate form (step 4) which cannot act as a substrate for the tRNA synthetases, but form the acid-soluble pool (P). AA in P can be displaced, dashed arrows in pathway 6. Usually further decay occurs (step 5) to the freed amino acid. This equilibrates with other extracellular or intracellular AA, and is free to recirculate, completing the flux pathway[7].

tion to occur and explains linear kinetics of incorporation (step 3). Unselected molecules decay, initially remaining unavailable for reselection (step 4) but maintaining a turnover of molecules through the system. These 'rejected' complexes form a sump - the acid-soluble pool - which is discharged by first order kinetics for all amino acids[18], the latter returning to the whole constellation of molecules in the system again. One explanation for the behavior of glycine is that the highly activated species produced at step 2 (fig. 4) is more stable than for other amino acids, as is step 4 (Inglis and Wheatley[19,20]).

The acid-soluble pool for all amino acids, including glycine and proline, is on the efflux side of the protein polymerising system. It provides the flux which offers a continuous selection of available amino acids to ensure precise requirements for protein synthesis can be obtained. This automatic system increases the chances of each amino acid being obtained in its correct proportion despite considerable nutritional (medium) imbalance[17]. The data is inconsistent with postulated cell membrane transport systems, especially for the Na^+-dependent nonessential amino acids (A, Gly and ASC systems) since this could not account for the instantaneous redistribution of precursor required for immediate linear incorporation. Only by tRNA molecules being charged directly across the cell membrane could the kinetics of incorporation be explained as adequately as in figure 4. This possibility is reviewed in a broader context by Henshaw[21], but has little evidence to support it.

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