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EVIDENCE FOR THE SELECTION BY THE MEMBRANE TRANSPORT SYSTEM OF INTRACELLULAR OR EXTRACELLULAR AMINO ACIDS FOR PROTEIN SYNTHESIS

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SUMMARY

Comparisons were made of the utilization of intracellular and extracellular proline for protein synthesis *in vitro* by embryonic chick cartilage and of the effects on these processes of agents known to modify proline uptake (serum, ouabain, amino acids).

The results support the concepts that a complex formed between the amino acid and the membrane transport system is the source of substrate for activation and incorporation into protein or, alternatively, for release intracellularly; that in order for intracellular amino acid to be incorporated, it must first become reassociated with the membrane, in competition with extracellular amino acids and at a site exposed to other extracellular modifiers of amino acid uptake; and that serum stimulates the incorporation and/or intracellular accumulation of amino acids by stimulating the formation of the membrane–amino acid complex.

INTRODUCTION

The assumption that amino acids for protein synthesis are drawn from the intracellular pool of free amino acids has appeared to some workers to be incompatible with observation. When various tissues are incubated *in vitro* with radioactive amino acids, the specific activity of the intracellular pool increases in a curvilinear manner for some hours while the rate of incorporation of radioactivity into protein is typically linear from almost the beginning of the incubation. To explain this phenomenon it has been hypothesized that intracellular free amino acids are functionally compartmented into two or more distinct pools[1–4] with a small rapidly equilibrated one being the protein precursor pool. Alternatively, it has been proposed that free amino acids for protein synthesis are drawn from the extracellular pool, with some of the incoming amino acids being diverted in the membrane for protein synthesis instead of being transferred to the intracellular pool[5–7].

Earlier work[8] with embryonic chick cartilage *in vitro* had indicated that in this tissue the rate of protein synthesis is limited by the rate of amino acid transport. We have used this system for further investigation of the relationship between these two processes. The experiments reported here were designed to compare the utilization

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for protein synthesis of extracellular and intracellular proline, in the presence or absence of factors which stimulate or inhibit proline uptake and incorporation.

The results also bear on the question of whether the stimulation by serum sulfation factor of both amino acid transport and protein synthesis could be accomplished by action at a single membrane site.

METHODS

Tissue. Fertile eggs (White Leghorn–Black Austrolorpe cross) were obtained commercially. The tissue used was the intact cartilaginous pelvic bone of the 12-day embryo. These rudiments (two per embryo) are thin plates with a wet weight of approximately 10 mg and are only slightly mineralized.

Conditions of incubation. Each incubation vial contained four bones and 4 ml of medium. The standard incubation medium used (pH 7.40–7.45) was Dulbecco phosphate buffer (which contains sodium, potassium, magnesium and calcium chlorides) *plus* Na_2SO_4 (0.3 mM) and glucose (1 mg/ml). Amino acids were added individually as noted in the legends or as a standard amino acid mixture. This mixture consisted of Eagle's minimum essential amino acid mixture[9] *plus* glutamine (1 mM) and proline, glycine and serine (each 0.2 mM). The essential amino acid mixture was sometimes used at one-half the recommended concentration. Radioactive amino acids were added as required.

The stoppered vials were incubated with shaking in a 37 °C water bath. In some experiments one vial was held at 0 °C instead of 37 °C in order to provide a measure of extracellular radioactivity for use as a blank correction[10].

Separation of amino acid fractions. To terminate the incubation, each bone was removed from the medium, blotted, and dropped into a scintillation counting vial which contained 2 ml of cold 0.9 % NaCl solution. The vial was maintained at 0 °C, with occasional shaking, for 20 min for extraction of the extracellular fraction (Fraction E). The bone was then removed from the vial, blotted, and placed on a labelled piece of parafilm. After being allowed to dry overnight at room temperature, it was weighed to the nearest microgram. It was then placed in a second counting vial with 2 ml of water and re-extracted with occasional shaking at room temperature for 2 h. This second extract is referred to as the free intracellular fraction (Fraction I). After the water extraction, the bone was blotted and transferred to a third counting vial. The bone was allowed to dry, 0.2 ml of 5 M KOH was added and the vial was capped and allowed to stand overnight at room temperature. Final disintegration of the bone was achieved with the help of a vortex mixer. This KOH digest included the amino acids incorporated into protein and is referred to as Fraction P.

Radioactivity was measured in all samples by liquid scintillation counting as previously described[10]. Results were calculated as μmoles of substrate amino acid per g dry weight of bone. For this purpose, it was assumed that the specific activity of amino acid in the various fractions was the same as that of the medium, with no dilution by endogenous non-radioactive proline. To the extent that there was such dilution, the calculated amounts are underestimated.

The tables and figures show the mean values for the four bones of each vial (unless another n is indicated) and the standard deviations ($[\Sigma d^2 \div (n-1)]^{\frac{1}{2}}$).

The fraction designations are based on earlier work with this tissue[10] in which

it was shown that after incubation with labelled amino acid, the radioactivity in the water extract of the dried bone (Fraction E *plus* Fraction I, which was referred to as the "diffusible fraction") consisted for the most part of unchanged substrate amino acids, although, for longer incubations, this fraction also contains a minor component of protein. The radioactivity left in the tissue after the water extraction was shown to be precipitable by trichloroacetic acid. Furthermore, the appearance of radioactivity in this unextracted fraction was promptly inhibited if the incubation medium contained puromycin, in contrast to the delayed puromycin effect on intracellular accumulation[11]. Therefore, Fraction P is usable as a measure of amino acid incorporation into protein.

RESULTS

Extraction of the extracellular fraction (*Fig. 1*). Other workers[12–14] have concluded that after intracellular accumulation of radioactive amino acid by muscle, the extracellular radioactivity can be removed from the tissue without major loss from the intracellular fraction by extraction in the cold. As can be seen from Fig. 1, such a fractionation is also possible with the intact cartilaginous rudiments used here. In this experiment, ^{3}H-labelled α-aminoisobutyric acid was preaccumulated in the bones before the latter were transferred to 0.9 % NaCl solution in an ice bath. Fraction E was obtained by extraction for periods ranging from 10 min to 3 h. The time courses of the appearance of α-aminoisobutyric acid in the extracellular extract (Fraction E) and of its compensatory loss from the subsequently extracted intracellular fraction (Fraction I) indicate that the procedure does not provide complete separation of these two amino acid pools. However, it is apparent that the bulk of Fraction E can be thus simply separated from most of the free intracellular pool.

Fig 1 also illustrates the fact, previously reported for muscle by Hider *et al.*[14], that after accumulation of concentrated free intracellular pools of radioactive amino acid, the radioactive amino acid content of the "extracellular" fraction can exceed that to be expected on the basis of extracellular water content. For proline and α-aminoisobutyric acid, we find that the inulin space (at 0 °C or 37 °C) and labelled amino acid space (at 0 °C) are only slightly different. On incubation at 37 °C, however, the content of labelled amino acid in Fraction E increases slowly during the period when the content in Fraction I increases (see also Fig. 2).

Early time course of accumulation of isotope in Fractions E, P and I (*Fig. 2*). To compare the initial rates of accumulation of extracellular [^{3}H]proline in Fractions E, I and P the experiment shown in Fig. 2 was done. There was a relatively slight increase of ^{3}H in Fraction E during the 30-min incubation. The increase of isotope in Fraction P was linear after 10 min. There is a typical delay of 5–10 min (as seen in Fig. 2) before any isotope can be detected in Fraction P, but thereafter we do not observe significant departures from linearity. In contrast, radioactivity accumulates in Fraction I at a decreasing rate. With longer times of incubation, this curve reaches a plateau in 2–3 h (see, for example, Fig. 3).

The implications of this set of curves are that about 7 min are required for [^{3}H]proline to be transferred in detectable amounts from the incubation medium into the protein fraction; that the specific activity of proline in Fraction I would have been changing during the continual accumulation of radioactive proline into an

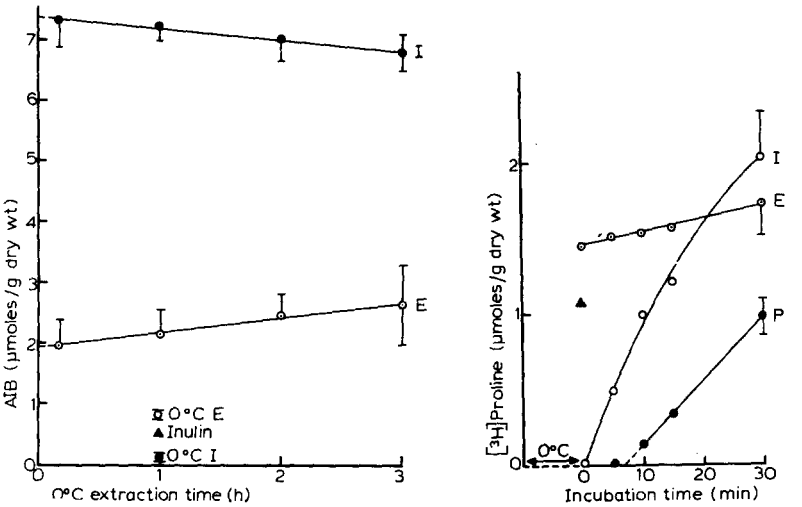

Fig. 1. Extraction at 0 °C of tissue extracellular α-aminoisobutyric acid. All bones were incubated for 2 h at 37 °C in medium containing 0.1 mM ^{3}H-labelled α-aminoisobutyric acid and no other amino acids. Extracellular radioactivity (E) was separated using extraction periods of 10 min, 1, 2 or 3 h in cold saline. The radioactivity remaining in the bones after extraction at 0 °C was determined and calculated as free intracellular α-aminoisobutyric acid (I). Also shown, for 1-h extraction, are α-aminoisobutyric acid in Fractions E and I when the preliminary incubation was at 0 °C instead of 37 °C. The calculated amount of extracellular radioactivity, based on the specific activity of the medium and the previously determined inulin space, is indicated (▲) in this and subsequent figures.

Fig. 2. Accumulation of [^{3}H]proline in extracellular (E), free intracellular (I), and protein (P) fractions. The incubation medium contained insulin (1 mU/ml), [^{3}H]proline (0.2 mM) and the complete amino acid mixture. Bones were held in the medium for 45 min at 0 °C before being transferred to 37 °C for the times indicated. Inulin space is indicated (▲).

initially unlabelled intracellular pool; that the [^{3}H]proline incorporated into protein was drawn from a pool of constant specific activity; that the specific activity of proline in Fraction E may have been constant, or nearly so; and that, therefore, Fraction E may have been the precursor pool for Fraction P, although Fraction I could not have been.

The incorporation of isotopic proline from differentially labelled intracellular and extracellular pools (Fig. 3). To compare the utilization of extracellular and intracellular proline for protein synthesis, we adopted the approach used by Hider *et al.*[7]. The bones were incubated initially with [^{14}C]proline and were then transferred to a second medium of identical composition except that the proline of the second medium was labelled with ^{3}H. The increase of ^{14}C in Fraction P ceased completely on removal of the bones from the [^{14}C]proline medium (Fig. 3). The appearance of [^{3}H]proline in Fraction P, on the other hand, appeared to commence promptly after the transfer and at a constant rate which was as great as the prior rate of incorporation of [^{14}C]-proline, even though initially the concentration of [^{3}H]proline in Fraction I would have been low compared to that of [^{14}C]proline.

The effects of serum, ouabain and extracellular metabolizable amino acids on the incorporation of preaccumulated proline (Fig. 4, Tables I–III). The above experiments support the conclusion that proline for protein synthesis is drawn from the extra-

cellular pool, when available, rather than from the supply of intracellular free proline. It therefore seemed reasonable to speculate that intracellular proline, in order to be incorporated, must first become reassociated with the membrane transport system to be re-presented for activation and incorporation into protein. If this is indeed the route taken by intracellular amino acid for incorporation, then this incorporation may be directly susceptible to extracellular agents which influence amino acid transport. We accordingly attempted to assess the effects on the incorporation of pre-accumulated intracellular proline of agents known to inhibit or stimulate the uptake of extracellular proline.

Stimulation by serum of incorporation of proline from the incubation medium is shown in Fig. 4. The incorporation of proline was again linear with time (after the usual lag) with or without serum. Under the conditions of this experiment, the ^{14}C content of Fractions E and I was not changed by serum.

To assess the effects of serum and of the extracellular proline on the incorporation of intracellular proline, [^{14}C]proline was preaccumulated. The bones were then transferred to a secondary incubation medium containing non-radioactive proline

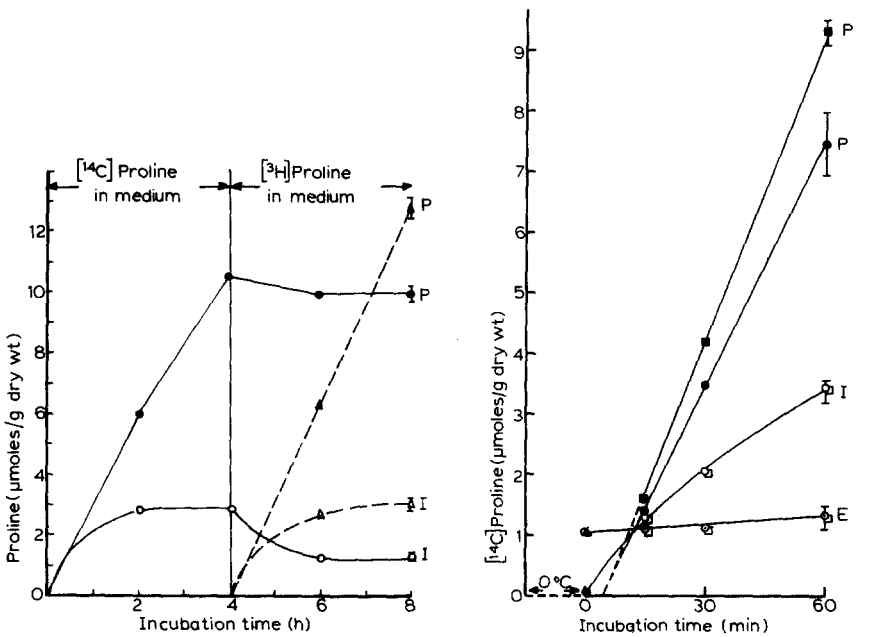

Fig. 3. Proline incorporation during and after accumulation of a labelled intracellular pool. The medium contained the complete amino acid mixture (at half strength) and [^{3}H]- or [^{14}C]proline (0.2 mM). Bones were incubated first with the [^{14}C]proline medium for 2 or 4 h. At 4 h, bones from some vials were blotted and transferred to [^{3}H]proline and the incubation was continued for an additional 2 or 4 h. In these bones both ^{3}H (▲---▲) and ^{14}C (●—●) were determined in the free intracellular (I) and the protein (P) fractions. Extracellular radioactivity was not measured. Instead, Fraction I was obtained by use of a 0 °C blank (see Methods).

Fig. 4. Effect of serum on accumulation of [^{14}C]proline in tissue fractions. The incubation medium contained [^{14}C]proline (0.2 mM), the complete amino acid mixture, and insulin (1 mU/ml). The bones were preincubated in the medium at 0 °C for 30 min before transfer to 37 °C. Some vials (□, ■) contained normal human blood serum (7.5 %). Inulin space is shown (▲).

or no proline at all. Serum was included in the secondary incubation medium in some cases. The ^{14}C content of Fractions E, P and I after the secondary incubation, and the changes in these fractions from the initial values, are shown in Table I.

The presence of non-radioactive proline in the medium did not modify the 72 % loss of label from Fraction E. However, it appeared to have caused a slightly greater retention of preaccumulated [^{14}C]proline in Fraction I.

Preaccumulated [^{14}C]proline was incorporated into Fraction P when extracellular proline was not available. This incorporation of preaccumulated label (in the absence of extracellular proline) was at a greatly reduced rate as compared to the hourly rate of incorporation during the initial incubation with extracellular [^{14}C]proline (1.7 *vs* 7.9 μmoles/g dry wt per h). Other experiments have shown that this decreased rate of incorporation of preaccumulated radioactivity is constant for at least 1 h (with or without serum) rather than being a temporary continuation at a faster rate followed by a cessation or slowing of incorporation during the period observed. However, even this low incorporation of preaccumulated radioactivity was prevented by the presence of proline in the secondary medium.

As can be seen from the last columns of Table I, in the absence of extracellular proline serum caused a 100 % stimulation of the incorporation of preaccumulated proline. However, in the presence of extracellular proline, there was no incorporation of preaccumulated proline, even in the presence of serum.

In another experiment, intracellular and extracellular proline were again differentially labelled. The bones were incubated first with medium containing [^{14}C]proline, but no other amino acids. The other amino acids were omitted in order to minimize proline incorporation and allow greater accumulation of [^{14}C]proline in Fraction I during the initial incubation. The bones were then transferred to secondary media which contained the complete amino acid mix (*plus* or *minus* serum

TABLE I

THE EFFECT OF EXTRACELLULAR PROLINE AND SERUM ON THE INCORPORATION OF PREACCUMULATED INTRACELLULAR PROLINE

All bones were incubated for 3 h in the same [^{14}C]proline medium as for the experiment of Fig. 4. The incubation of two vials (eight bones) was then terminated for the determination of initial (preaccumulated) tissue proline radioactivity. The remaining bones were incubated for an additional hour in fresh medium which contained no [^{14}C]proline and did or did not contain [^{12}C]-proline or normal human serum (7.5 %). [^{14}C]proline content of the different fractions after this additional hour (and the changes from the initial values) are shown as μmoles/g dry wt ± S.D.

Tissue fraction	*Serum*	^{14}C *after 1 h*		*Change*	
		−Proline	*+Proline*	*−Proline*	*+Proline*
Extracellular (E)	No	0.42 ± 0.06	0.42 ± 0.14	−1.02	−1.02
(Initial: 1.44)	Yes	0.45 ± 0.04	0.38 ± 0.07	−0.99	−1.06
Intracellular (I)	No	1.63 ± 0.24	2.21 ± 0.13	−4.97	−4.39
(Initial: 6.60)	Yes	1.61 ± 0.25	2.08 ± 0.19	−4.99	−4.52
Protein (P)	No	25.5 ± 1.7	23.4 ± 1.6	+1.7	−0.4
(Initial: 23.8)	Yes	27.2 ± 0.8	24.0 ± 1.9	+3.4	+0.2
Total	No	27.6	26.0	−4.2	−5.8
(Initial: 31.8)	Yes	29.3	26.5	−2.5	−5.3

TABLE II

THE EFFECTS OF SERUM AND OUABAIN ON CHANGES IN TISSUE FRACTIONS OF PREACCUMULATED AND EXTRACELLULAR PROLINE

Bones were incubated for 3 h in medium containing no amino acids except [^{14}C]proline (0.2 mM). They were then transferred to medium (at 0 °C) which contained [^{3}H]proline (instead of [^{14}C]proline), the complete amino acid mixture, and in some cases, normal human serum (7.5%), ouabain (0.1 mM), or both. The bones were held in the secondary media at 0 °C for 40 min. In the case of two vials (eight bones), the incubation was terminated at this point in order to obtain initial values. The other bones were transferred to 37 °C for a secondary incubation of 1 h. The changes during this additional hour in the ^{3}H and ^{14}C content of the different fractions are shown as μmoles/g dry wt ± S.D.

	Extracellular		*Intracellular*		*Protein*		*Total*	
	— Proline	*+ Proline*	*— Proline*	*+ Proline*	*— Proline*	*+ Proline*	*— Proline*	*+ Proline*
Δ ^{14}C (from tissue)								
Control	−3.7	−4.0	−13.5	−13.6	+2.8	+1.2	−14.4	−16.4
+ Serum	−4.2	−4.0	−14.1	−14.0	+3.3	+1.4	−15.0	−16.6
+ Ouabain	−4.1	−4.3	−14.7	−15.1	+0.8	+0.6	−18.0	−18.8
+ Both	−4.7	−4.1	−14.6	−15.0	−0.5	−0.1	−19.8	−19.2
Δ ^{3}H (from medium)								
Control		+1.8		+ 4.3		+2.0		+ 8.1
+ Serum		+1.6		+ 3.4		+3.1		+ 8.1
+ Ouabain		−0.1		− 0.4		+0.6		+ 0.1
+ Both		−0.2		− 0.4		+0.1		− 0.5
Initial values								
[^{14}C]Proline	4.7 ± 0.9		16.3 ± 2.1		6.3 ± 0.3		27.3	
[^{3}H]Proline	2.4 ± 0.2		1.1 ± 0.4		0.2 ± 0.1		3.7	

and/or ouabain) with or without proline which, when present, was tritiated. The changes of ^{14}C and ^{3}H in Fractions E, P and I during the secondary incubation of 1 h are shown in Table II.

In this experiment, too, more of the preaccumulated ^{14}C was transferred to Fraction P in the absence of medium proline than in its presence, so that the total loss of ^{14}C from the tissue was less if the medium did not contain proline. In the absence of medium proline, serum in this experiment caused only an 18 % stimulation of incorporation of preaccumulated proline (*vs* 11 % in the presence of medium proline). By contrast, it simultaneously caused a 55 % stimulation of incorporation of [^{3}H]proline when it was present in the medium. This latter stimulation occurred without a stimulation of total uptake by the tissue of [^{3}H]proline.

In this experiment, the initial concentrations of ^{14}C in Fractions E and I were markedly higher than those of the experiment of Table I. With these higher levels, the rate of incorporation of preaccumulated proline was greater than was that of the medium proline (2.8 *vs* 2.0) and incorporation of tissue proline continued, although at an inhibited rate, after the transfer to the medium containing extracellular proline. This observation – that the inhibitory effects of medium proline on the incorporation of tissue proline can be at least partially overcome if the concentration of preaccumulated proline is increased – has been repeatedly confirmed, supporting the interpretation that intracellular and extracellular proline are in a competitive relationship with respect to protein synthesis.

In this experiment the effects of ouabain were also followed. It had little effect on the loss from Fractions E and I of preaccumulated [^{14}C]proline, but it strongly inhibited the incorporation of preaccumulated ^{14}C into Fraction P. With respect to medium [^{3}H]proline, ouabain prevented its accumulation in Fraction I, prevented the increase of Fraction E above the 0 °C value, and strongly inhibited (but did not prevent) incorporation into Fraction P. The inhibition by ouabain of the transfer of proline from the medium to the free intracellular pool was 100 %, whereas the inhibition of incorporation of proline whether from the tissue or from the medium was 70 %. This greater inhibition by ouabain of the appearance of medium proline in Fraction I than in Fraction P has been observed during incubations lasting from 30 min to 3 h.

The stimulation by serum of intracellular and extracellular proline incorporation was not apparent in the presence of ouabain. Indeed, in both cases there is an indication that serum accentuated the inhibition by ouabain.

It was possible that the inhibition by ouabain of incorporation of preaccumulated proline was indirect, resulting from inhibition of uptake of other amino acids which were present in the medium. If the uptake of those amino acids, rather than the supply of preaccumulated proline, were the limiting process for proline incorporation then ouabain would indirectly inhibit the incorporation of intracellular proline. This possibility seems unlikely in view of the results of Table III. For this experiment, [^{14}C]proline as well as a partial mixture of amino acids, including those (Group A) whose uptake might be expected to be inhibited by ouabain, and which would compete with proline for transport, were preaccumulated during a primary incubation. The bones were then incubated for an additional hour in non-radioactive secondary media containing various combinations of amino acids. Changes, during the final hour, of ^{14}C contents of Fractions I and P are shown.

Incorporation of preaccumulated [^{14}C]proline into Fraction P was greatest if the secondary medium contained the complete amino acid mixture without proline (Medium 6), whereas if the medium contained Group B or Group A alone, incorporation was less than in the complete absence of added extracellular amino acid. Ouabain caused little or no increase in the inhibition caused by proline alone, or Group A alone. However, it did inhibit incorporation of preaccumulated proline in the complete absence of extracellular amino acids (Medium 1) or when the medium contained Group B with or without Group A (Media 2, 5, 6). Although the competitive interrelationships in this type of experiment are complex, it is apparent that ouabain or extracellular competitors for proline transport can inhibit the incorporation of preaccumulated proline independently of any effect on the uptake of other extracellular amino acids needed for protein synthesis.

With all media, ouabain caused an increased loss of ^{14}C from Fraction I. This was presumably because it blocked the active process by which effluxed proline can be reaccumulated. Retention of ^{14}C in Fraction I was greatest in the absence of both ouabain and extracellular competitors (Medium 1).

The relative inhibition by α-aminoisobutyric acid of the incorporation of preaccumulated and extracellular proline (Table IV). If intracellular proline must return to the membrane transport system before incorporation, then extracellular α-aminoisobutyric acid (a competitive inhibitor of proline uptake) should inhibit the incorporation of preaccumulated intracellular proline. This prediction has been confirmed. For the experiment shown in Table IV, [^{14}C]proline was preaccumulated. Bones were then transferred to secondary media containing [^{3}H]proline or no proline at all. Some of the secondary media also contained α-aminoisobutyric acid. In order

TABLE III

THE EFFECT OF EXTRACELLULAR AMINO ACIDS ON LOSS AND INCORPORATION OF PREACCUMULATED PROLINE

During a primary incubation of 3 h, the medium contained [^{14}C]proline (0.2 mM) and non-radioactive amino acids of Group A (serine, valine, glycine, histidine, methionine, threonine, arginine and lysine) at the same concentrations as in the standard amino acid mixture. Bones were then blotted and transferred to non-radioactive secondary media containing, in some cases, ouabain (0.1 mM) and amino acids as indicated. Group B consisted of the rest of the standard amino acids (glutamine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan and cystine). Initial [^{14}C]-proline fractions were determined at the time of transfer from the primary medium and were 10.1 ± 0.9 and 4.9 ± 0.4 for Fractions I and P, respectively. The changes in Fractions I and P during the secondary incubation are shown as μmoles/g dry wt ± S.D. The initial value for Fraction E was 1.72 ± 0.25. Decreases in this fraction were essentially the same for all treatments and are not shown, although, in every case, loss in the presence of ouabain was slightly greater than in its absence. For the 6 media, the average [^{14}C]proline remaining in Fraction E after the secondary incubation, was 0.57 (range 0.48–0.70) in the absence, and 0.47 (range 0.31–0.56) in the presence of ouabain.

Medium	*Amino acids in medium*			*Δ^{14}C Fraction I*		*Δ^{14}C Fraction P*	
	Group B	*Group A*	*Proline*	*−Ouabain*	*+ Ouabain*	*−Ouabain*	*+ Ouabain*
1	−	−	−	−6.0 ± 0.3	−7.5 ± 0.2	+2.1 ± 0.8	+1.2 ± 0.4
2	+	−	−	−7.6 ± 0.5	−8.6 ± 0.2	+1.7 ± 0.4	+1.2 ± 0.3
3	−	+	−	−6.7 ± 0.7	−8.2 ± 0.2	+1.6 ± 0.6	+1.3 ± 0.3
4	−	−	+	−7.2 ± 0.3	−8.8 ± 0.5	+1.1 ± 0.2	+1.0 ± 0.2
5	+	+	+	−7.6 ± 0.3	−8.6 ± 0.3	+1.5 ± 0.7	+0.6 ± 0.2
6	+	+	−	−8.0 ± 0.6	−8.6 ± 0.1	+2.6 ± 0.5	+1.1 ± 0.3

TABLE IV

INHIBITION BY EXTRACELLULAR α-AMINOISOBUTYRIC ACID OF THE INCORPORATION OF INTRACELLULAR PROLINE

Bones were incubated for 3 h in medium containing insulin (1 mU/ml) and [^{14}C]proline (0.2 mM) but no other amino acids. For two vials (eight bones), the incubation was then terminated to obtain ^{14}C initial values. These were 6.8 ± 0.4 for Fraction P and 15.1 ± 2.1 for Fraction I. The other bones were blotted and transferred to secondary media which contained [^{3}H]proline (0.2 mM), or no proline at all. α-Aminoisobutyric acid (AIB) was also present in some cases, as indicated. After 1 h at 37 °C in the secondary media, the incubation was terminated. Changes in the ^{14}C and ^{3}H content of Fractions P and I during the last hour are shown, as μmoles/g dry wt ± S.D.

Medium proline:	*None*	*[^{3}H]Proline*		
Source of label:	*From tissue (^{14}C)*	*From medium (^{3}H)*	*From tissue (^{14}C)*	*Tissue plus medium*
Competitor		*Δ Fraction P*		
None	+ 2.7 ± 0.6	+1.26 ± 0.13	+ 1.6 ± 0.9	+2.9
AIB (0.5 mM)	+ 1.8 ± 0.4	+1.15 ± 0.06	+ 1.5 ± 0.5	+2.7
AIB (2.5 mM)	+ 1.2 ± 0.3	+0.79 ± 0.12	+ 1.1 ± 0.6	+1.9
		Δ Fraction I		
None	− 8.9 ± 0.4	+9.2 ± 1.0	− 9.7 ± 0.4	−0.5
AIB (0.5 mM)	− 9.3 ± 0.6	+6.9 ± 0.7	− 9.9 ± 0.4	−3.0
AIB (2.5 mM)	−10.9 ± 0.2	+3.6 ± 0.3	−10.7 ± 0.3	−7.1

to minimize competitive interactions, no other amino acids were added to either the primary or secondary incubation media. Under such conditions, proline incorporation still continues at a constant though diminished rate.

During the secondary incubation, incorporation of preaccumulated [^{14}C]proline, in the absence of medium proline, continued at approximately the same rate as during the initial incubation with medium [^{14}C]proline. α-Aminoisobutyric acid (2.5 mM) inhibited this incorporation of preaccumulated proline by 1.5 μmoles/g dry wt or 56 %. This same concentration of α-aminoisobutyric acid also inhibited the incorporation of [^{3}H]proline when it was present in the medium but the inhibition (0.5 μmoles/g or 37 %) was less severe. This greater inhibition by α-aminoisobutyric acid of the incorporation of preaccumulated as compared to medium proline was also apparent at the lower α-aminoisobutyric acid concentration (33 % *vs* 9 %) and has been confirmed in other experiments.

Proline in the medium also inhibited the incorporation of preaccumulated [^{14}C]proline (by 1.1 μmoles/g) with the decrease being replaced by an approximately equal incorporation of [^{3}H]proline from the medium (1.3 μmoles/g). When both proline and α-aminoisobutyric acid were in the medium, the combined inhibition of incorporation of preaccumulated proline was no greater than that caused by α-aminoisobutyric acid alone in the absence of medium proline.

The inhibition by α-aminoisobutyric acid of the intracellular accumulation of [^{3}H]proline was markedly greater than its inhibition of [^{3}H]proline incorporation. This is to be expected if one assumes that incoming proline is available for incorporation before it is released intracellularly[15].

DISCUSSION

We believe that the results presented above are most easily explained by the following set of hypotheses, some of which have been previously suggested by others.

(1) The selection of amino acids for protein synthesis occurs while they are in association with the "transport" system of cell membranes.

(2) The membrane–amino acid complex can also dissociate to release the amino acid into the intracellular pool. But because the incoming amino acid is made available for incorporation into protein before it is released intracellularly, if the formation of the complex is inhibited (as by ouabain or by transport competitors), the intracellular accumulation of amino acid can be more severely depressed than is its incorporation into protein.

(3) Amino acid which has been released intracellularly can return to be once again available for incorporation by reassociation at the "transport" membrane. There it is once again vulnerable to extracellular competitors, inhibitors, and stimulators of amino acid transport. In the case of proline, the effect of this is that extracellular proline has a competitive advantage for incorporation as compared to free intracellular proline.

(4) Serum stimulates the formation of the membrane–amino acid complex. Whether this action causes a stimulation of incorporation, of intracellular accumulation, or both, will depend upon whether and to what extent the protein synthetic process accepts the increased supply of substrate.

It is apparent that a transport–incorporation–accumulation system with the interrelationships postulated above would be an economical one, causing decreased utilization of intracellular amino acids during times of extracellular abundance. Such a system can reconcile reports that external amino acid concentrations regulate protein synthetic rates[16–18,4] and that amino acids can be incorporated directly from the extracellular pool[7,14] with others which indicate preferential incorporation of some amino acids from cellular sources[19,20] or detect correlations between rates of transport and incorporation[21,22,28].

An implication of the first two of the above hypotheses is that extracellular amino acids can be a more direct source of free amino acids for protein synthesis than free intracellular amino acids. This suggestion has been previously stated by others[5–7]. It receives support from most of the observations reported above. The more severe inhibition by ouabain or transport competitors of intracellular accumulation than of incorporation is a typical observation. The rates of accumulation in the protein and free intracellular fractions of labelled proline from the medium (Figs 2–4) preclude the possibility that the total free intracellular pool is the source of proline for protein synthesis.

The accumulation in this tissue of an apparently extracellular fraction of radioactive proline in excess of the amount to be expected in the extracellular water is analogous to a similar accumulation reported by Hider *et al.*[14] for glycine and leucine in muscle. From measurements of specific radioactivities they concluded that the extracellular amino acid, unlike the free intracellular pool, was a suitable precursor pool for protein.

This conclusion is further reinforced by the observation that the isotopic label of extracellular rather than intracellular proline was incorporated into protein if

both were available at approximately physiological concentrations. Upon the addition of extracellular proline, there was no detectable transition period in its displacement of preaccumulated proline for protein synthesis, despite the fact that displacement of the preaccumulated label in the free intracellular pool was gradual and incomplete. Similar demonstrations of the preferential use of extracellular glycine and leucine for protein synthesis by muscle were previously reported by Hider *et al.*[7,14].

On the other hand, Righetti *et al.*[20] reported that much of the amino acid used for ferritin synthesis by HeLa cells was derived from cellular sources. If the hypotheses stated above are correct, both intracellular and extracellular sources may supply amino acids for protein synthesis; the choice will depend upon the relative affinities of membrane transport sites for the particular amino acids and the influence of the prevailing internal and external environments and concentrations. The fact that intracellular amino acids can be utilized for protein synthesis and can displace extracellular ones for incorporation into protein need not be interpreted, as it was by Tsukada *et al.*[23], and by Manchester[24], as an indication that the displacement occurs at an intracellular site.

In our experience, only if labelled intracellular proline is preaccumulated to a much higher concentration than that of the medium proline, does its incorporation continue when proline is added to the medium. This observation supports the inference that the selection of extracellular or intracellular substrate has a competitive basis, in accord with the third hypothesis above.

Other evidence that intracellular amino acid becomes reassociated with the transport membrane before incorporation is seen in the experiments showing that preaccumulation of proline does not shield its incorporation from the effects of extracellular inhibitors of amino acid transport. Ouabain, for example, which inhibits "transport ATPase"[25], inhibited the incorporation of preaccumulated proline as much as that of medium proline even in the absence of added extracellular maino acids. α-Aminoisobutyric acid competitively inhibits the uptake of proline, but is not itself incorporated or metabolized. Yet its inhibition of the incorporation of preaccumulated proline is greater than its inhibition of incorporation of proline from the medium.

Phang *et al.*[4], using embryonic rat bone, reported inhibition by α-aminoisobutyric acid of incorporation of proline from the medium. The observed inhibition was greater than that predicted by their model, for which the protein precursor pool was assumed to be a small compartment of intracellular amino acid. Their model did not take into account the possibility of re-exposure to transport inhibitors of intracellular proline *en route* to incorporation.

The evidence for the fourth hypothesis has as background a large number of earlier experiments which have attempted to determine whether certain hormones (insulin, growth hormone, serum sulfation factor) act at one site or two different sites to stimulate the two processes of protein synthesis and amino acid transport (for reviews, see refs 15, 26). Conclusions which have favoured one or two sites have implicitly rested on the assumption that the incorporation of amino acid follows its transport and release inside the cell. However, as has been already pointed out by Hendler (ref. 15, p. 284 *et seq.*), if intracellular accumulation follows rather than precedes the selection of amino acid for incorporation, a single site of hormone action again appears sufficient.

The results reported here with serum* are quite compatible with this concept. In these experiments, serum stimulated the incorporation of medium proline without altering its intracellular accumulation. In addition, the comparisons of serum effects on incorporation of intracellular and extracellular proline show that if no proline is in the medium, serum stimulates the incorporation of preaccumulated proline. If however, medium proline is present, serum stimulates the incorporation of the extracellular label while the effect on incorporation of intracellular label is decreased or eliminated. This observation appears to be analogous to the observation by Hider *et al.*[27] that, in muscle, insulin stimulates the incorporation of glycine, leucine or proline if the labelled amino acid is in the medium, but not if it has been preaccumulated and unlabelled amino acid is in the medium.

These results are rationalized if one assumes that serum (or insulin) acts at the membrane site for which extracellular and intracellular amino acids compete and from which amino acids are drawn for either protein synthesis or intracellular release.

If our interpretations are correct, past emphasis in experiment and discussion on the transport function of the membrane–amino acid interaction may have diverted attention from the significant effect of this interaction (amino acid selection for protein synthesis) to the incidental consequence (intracellular accumulation).

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* We assume that the effects of serum were due to the presence of serum sulfation factor and, in Table II, insulin. Both of these agents have been previously shown to stimulate protein synthesis in this tissue.

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