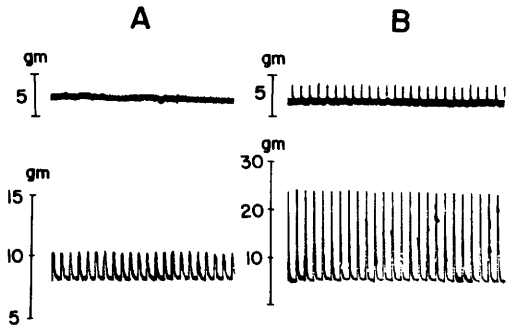

FIG. 1. Isometric twitch tension of m.ext.dig. longus of the rat one (upper traces) and three weeks (lower traces) after poisoning with botulinum toxin. In the right leg (records B) the nerve innervating the muscle was crushed at time of poisoning while the nerve to the muscle of the other leg (records A) was left intact. Electrical stimulation of sciatic nerve with single shocks every second.

terminals.

Summary. Results are presented showing that it is possible to restore neuromuscular transmission in botulinum paralyzed rat skeletal muscle by inducing the formation of new motor nerve terminals. The toxin, bound to the original endings, does not interfere with the regeneration following a crush of the motor nerve, and the new terminals can establish effective neuromuscular transmission after a few days. In muscles treated with the toxin alone the paralysis lasts for several weeks.

1. Bishop, G. H., Bronfenbrenner, J. J., *Am. J. Physiol.*, 1936, v117, 393.

2. Guyton, A. C., MacDonald, M. A., *Arch. Neurol. & Psychiat.*, 1947, v57, 578.

3. Lamanna, C., *Science*, 1959, v130, 763.

4. Thesleff, S., *J. Physiol.*, 1960, v151, 598.

5. Zacks, S. I., Metzger, J. F., Smith, C. W., Blumberg, J. M., *J. Neuropath. and Exp. Neurol.*, 1962, v21, 610.

Received February 18, 1964. P.S.E.B.M., 1964, v116.

The Flexible Nature of the Amino Acid Pool in L Strain Fibroblasts.* (29147)

ROBERT J. KUCHLER† (Introduced by R. C. Grauer)
Department of Microbiology, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.

Mammalian cells maintained or growing in tissue culture contain intracellular pools of free amino acids. The internal concentration of any amino acid is usually greater than the external concentration; this implies that a permeable barrier exists and that amino acids are actively transported into these cells. The essential amino acids in the pool must come from the external medium; however, non-essential amino acids are derived both from exogenous sources and endogenous synthesis. Regardless of the origin of the pooled amino acid, the internal level is dependent on the external concentration(1).

Previously, we showed that L strain fibroblasts have a greater capacity to accumulate non-essential than essential amino acids when both are present in the medium at approximately the same levels(2). This finding has led us to speculate that non-essential amino acids may interfere with the pooling of essential amino acids, so we proceeded to investigate the quantitative nature of the free amino acid pool during exponential growth when cells are cultured in a medium which omits the non-essential amino acids.

Materials and methods. A replicate series of L strain fibroblast cultures was set up in 100 milk dilution bottles in medium 199 supplemented with 0.5% bacto-peptone (199P). Ten milliliters of a suspension containing 2.5×10^5 L cells/ml was used as inoculum for each bottle. These cultures were incubated at 37°C in a stationary position for 36 hours, at which time the medium bathing the cells was decanted, and the cells

* Supported in part by Public Health Research Grant.

† Present address: Dept. of Bacteriology, Rutgers Univ., New Brunswick, N. J.

attached to the glass were washed with Hank's balanced salt solution (BSS) prewarmed to 37° C. Ten milliliters of fresh medium 199P were pipetted onto one-half the cultures and the remaining cell cultures received 10 ml of a medium containing only the essential amino acids supplemented with 5% dialyzed horse serum (MEMS). The respective media were changed at 24 hour intervals. The growth cycle was followed by sampling cultures in triplicate at various times. The attached cells were harvested by scraping them off the glass into the used medium with a rubber policeman. The cells were sedimented in a vaccine tube, and the packed cell volume was recorded. Deoxyribonucleic acid (DNA) and protein determinations were carried out on the cell masses(2).

Twenty cultures were selected randomly from each set at the 84th hour of the culture cycle. The cultures were harvested by scraping the cells into the bathing medium. The 10-ml suspensions in 199P were combined separately from those in MEMS. The cells in these 200-ml combined suspensions were sedimented at 300 × *g*. The media were removed from the 2 cell packs, and each was washed once with 40 ml of balanced salt solution. The cells were again sedimented, and the washing fluids removed. The cells were extracted with 5 ml of cold trichloroacetic acid (TCA); the free intracellular amino acids were dissolved in TCA, while the higher molecular weight materials were precipitated. The precipitates were extracted with 10 ml of hot TCA and 20 ml of ether. The final residues containing essentially all of the cell protein were dissolved by addition of 10 ml of 0.2 N NaOH. One milliliter from each protein suspension was mixed with 1 ml of 12 N HCl in a glass ampule. The glass ampules were sealed and autoclaved at 121°C for 18 hours. These protein hydrolysates, the cold TCA extracts, fresh media and used media were extracted with ether. All of the samples were adjusted to pH 2.5 and analyzed independently for their amino acid content by ion-exchange chromatography(3).

A similar 100 bottle replicate culture series as described above was set up in 199P medium. After 36 hours the medium was decanted from each bottle, and the cell monolayers were washed with BSS. This time 10 ml of MEMS was added to one-half the cultures and MEMS with twice the concentration of each amino acid was added to the remaining cultures. These media were changed again at the 60th hour, and the cell cultures were harvested into separate suspensions at the 84th hour. The cells were sedimented and washed with BSS. The free amino acids were extracted with cold TCA. Fresh media, used media and the free amino acid pools were quantitatively analyzed by ion exchange chromatography.

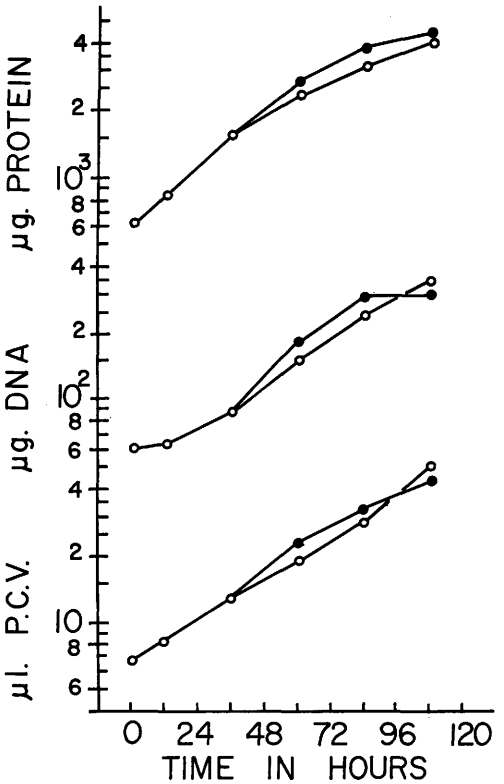

FIG. 1. Packed cell volume, DNA and protein in L cell populations growing in 199P and MEMS. (○) 199P, (●) MEMS.

Experimental results. Fig. 1 depicts the increases in packed cell volume, DNA and protein throughout the culture cycle in L strain populations. At zero time each replicate bottle culture contained approximately 7 µl of packed cells, 60 µg of DNA and 625 µg of protein. Thirty-six hours later sample cultures from the replicate series showed that

TABLE I. Amino Acid Levels in Fresh Media, Used Media and Cell Water in L Cell Populations Growing in 2 Media with Differing Amino Acid Concentrations.

	199 peptone medium (199P)				Minimum essential medium + 5% horse serum (MEMS)			
Amino acid	Fresh media conc (a) (mM)	Media conc at harvest (b) (mM)	Conc in cell water (c) (mM)	Conc ratio (d) —	Fresh media conc (e) (mM)	Media conc at harvest (f) (mM)	Conc in cell water (g) (mM)	Conc ratio (h) —
Aspartic acid	.57	.55	.63	1.1	—	.09	.62	6.9
Glutamine	Trace	Trace	Trace	—	.80	.30	3.30	11.0
Threonine	.67	.54	1.74	3.2	.39	.20	3.10	15.0
Serine	.96	.81	2.13	2.6	—	.08	.30	3.8
Glutamic acid	.89	.76	3.87	5.1	.21	.39	7.88	20.0
Proline	.35	.30	2.74	9.1	—	.04	.81	20.0
Glycine	.93	.86	9.99	11.6	—	.02	.38	19.0
Alanine	.91	.79	3.18	3.9	—	.21	3.19	15.7
Cystine	.13	—	Trace	—	.11	.01	Trace	—
Valine	.67	.51	.54	1.0	.34	.22	.83	3.9
Methionine	.28	.23	.30	1.3	.10	.06	.36	6.0
Isoleucine	.44	.34	.33	1.0	.21	.15	.54	3.6
Leucine	.98	.63	.66	1.0	.19	.06	.29	4.8
Tyrosine	.43	.35	.58	1.6	.10	.07	.52	7.4
Phenylalanine	.60	.46	.87	1.9	.17	.13	.85	6.1
Lysine	.88	.76	.48	.6	.23	.14	.29	2.0
Histidine	.18	.06	Trace	—	.14	.09	Trace	—
Arginine	.98	.91	.84	.9	.22	.14	.28	2.0
Ammonia	1.52	2.98	2.28	.8	—	1.17	3.18	2.0

each culture increased to an average of 12 μl of packed cells. By the 108th hour the packed cell volume increased to 36 μl, the DNA to 290 μg and the protein to 4400 μg in the populations grown in 199P. The increases in MEMS populations were comparable at this time; however, rate of cell growth was slightly enhanced when the medium was initially added to the cell cultures previously growing in 199P. The increased rate of growth was not maintained in MEMS grown cultures but reverted to the previous rate between the 60th and the 84th hour.

The fact that we could obtain uniform growth rates in 2 selected media provided us with the opportunity to compare the location and the quantity of each amino acid in the two culture systems under similar conditions. The complete data indicating the concentration of each amino acid in fresh media, media at harvest and in cell water are shown in Table I. Column "a" shows the amino acid concentrations in fresh medium 199P, and column "b" shows them when the cultures were harvested. The amino acid concentrations in the pool in cells grown in 199P are shown in column "c." It can be noted that all of the amino acids in 199P, originally and at harvest were detected in the pool. Some of the amino acids were concentrated by the cells in excess of the external level. Glycine, for example, was present in the pool at 9.99 mM which is a concentration 11 times greater than that of the bathing medium; it represents about 30% of the total amino acid content of the pool. Alanine, glutamic acid, proline and serine were each found to make up more than 10% of the amino acid pool, and each was concentrated over the exogenous level by a factor greater than three. Only threonine of all the essential amino acids was detected in excess of 10%. Each of the other essential amino acids constituted about 1% of the pool, and each was found at an intracellular concentration about equal to the external level.

The results of a group of parallel cultures growing in a medium containing only the minimum essential amino acids and harvested at the same point in the culture cycle is also shown in Table I. The minimum essential medium was a commercial preparation, and the quantitative analysis is given in column "e." The only non-essential amino acid de-

tected in the medium was glutamic acid. Column "f" shows the results of a quantitative amino acid analysis on the culture medium after cell growth for 24 hours. Most notable is the fact that the non-essential amino acids are now detected at low levels except for glutamic acid which is now found at 0.39 mM. The free amino acid pool extracted from the cells growing in this medium is shown in column "g." It is striking that the amino acid pool is quantitatively modified from that observed in the cells growing 199P. The pool is also qualitatively different from the amino acid complement noted in fresh MEMS; however, it does simulate the amino acid array observed in MEMS at harvest. Four amino acids constitute the bulk of the pool. Of the total free intracellular amino acids, glutamic acid constitutes 40%, alanine 12%, glutamine 10% and threonine 12%. Glutamic acid was accumulated to 7.88 mM and concentrated 20-fold. Alanine was found at 3.19 mM and concentrated 16-fold despite the fact that it was not originally present in the medium. Threonine was the most efficiently pooled essential amino acid; it was accumulated to 3.10 mM although it was present at 0.39 mM in MEMS. The remaining essential amino acids except for glutamine were found in the modified pool at a concentration similar to those observed when the cells were grown in 199P despite the fact that these amino acids were all present at lower concentrations in MEMS. Hence, the cells demonstrate an enhanced capacity to accumulate essential amino acids in the absence of high exogenous levels of non-essential amino acids. Although the non-essential amino acids were omitted from MEMS, they were detected in the modified pool, but for the most part at much lower levels. The situation of glutamine uptake is unique. It is considered to be an essential amino acid, but it is utilized in excess of any other amino acid, and it appears to move into many metabolic pathways. The role of glutamine in tissue culture has previously been investigated(4). Our results show that about 15% of the glutamine can be detected in the culture medium at harvest, and the intracellular concentration is 3.30 mM.

TABLE II. Amino Acid Levels in Fresh Media, Used Media and Cell Water in L Cell Populations Growing in MEMS Containing a Double Concentration of Each Essential Amino Acid.

Amino acid	Fresh media conc (mM)	Media conc at harvest (mM)	Conc in cell water (mM)	Conc ratio —
Aspartic acid	—	.09	1.10	12.2
Glutamine	1.60	.62	4.57	7.4
Threonine	.78	.55	5.01	9.2
Glutamic acid	.42	.48	9.84	20.4
Proline	—	.06	1.33	22.1
Glycine	—	.04	.48	12.0
Alanine	—	.41	7.52	18.3
Cystine	.22	.03	Trace	Trace
Valine	.68	.53	1.08	2.0
Methionine	.20	.12	.36	3.0
Isoleucine	.42	.35	.99	2.8
Leucine	.38	.21	.56	2.7
Tyrosine	.20	.15	.59	4.2
Phenylalanine	.34	.26	.68	2.6
Serine, Histidine, Lysine, Arginine	Not quantitatively analyzed in this exp			

In another experiment we have noted that the pool could be modified in less than one generation. In fact, simply by changing the medium a dramatic alteration was observed, but approximately one generation was required to dilute out the pool which was present from the 199P under the conditions of our experiments.

The intracellular amino acid levels in 199P and MEMS were apparently optimal for L cell growth, since the rate of protein synthesis was not enhanced by doubling the concentration of each essential amino acid in MEMS. However, Table II shows that the pool levels for most of the amino acids were raised when the exogenous levels were increased. This again emphasizes that the amount of each amino acid in the pool is dependent upon the concentration in the medium, but it does not follow that the internal level is doubled for each as one might suspect if the pool level is a constant and independent function for each amino acid. The results imply that there are cellular controls for the accumulation of amino acids based on transport reaction, internal transformation and biosynthesis.

The residual protein from the precipitated masses remaining after the pools were extracted from the cells growing in 199P and

TABLE III. Percentage of Each Amino Acid in Protein Hydrolysates of L Cells Grown in 199P and MEMS.

	199P % of total	MEMS % of total
Aspartic acid	9.8	9.6
Threonine	4.6	4.9
Serine	6.4	5.8
Glutamic acid	13.4	12.6
Proline	7.1	6.8
Glycine	9.0	8.1
Alanine	8.8	8.3
Cystine	.6	.8
Valine	6.1	6.5
Methionine	1.9	2.1
Isoleucine	4.6	5.8
Leucine	9.2	9.7
Tyrosine	2.8	2.2
Phenylalanine	3.3	3.4
Lysine	4.7	5.2
Histidine	2.2	2.4
Arginine	5.5	5.8

MEMS were washed 3 times to insure that all of the free amino acids were removed. These protein precipitates were then hydrolyzed. Table III shows that the percentage of each amino acid in cell protein was similar for cells from either medium. Evidently, the medium change does not alter the composition of the proteins synthesized, despite the rapid alteration in the pool.

Discussion. The eighty-fourth hour has been selected as the time for comparing the free amino acid pools in presence and in absence of exogenous non-essential amino acids. This time is appropriate since the cell populations are growing exponentially and synthesizing proteins at similar rates. Under these conditions, amino acids would be removed from the pool at similar rates in the two systems, so that the transient intracellular free amino acids would depend only upon those conditions concerned with pool accumulation. The 36th hour has been chosen as the time to initiate the experiment since it is the beginning of the logarithmic phase; as this time the cells appear to be in a state of balanced growth(5). Such an approach allows a medium change at a subsequent 24 hour time interval in order to dilute out any amino acid residues remaining from the original bathing medium which contained a full complement of non-essential amino acids. In addition, more than one generation did occur after the first medium change, so that any excess pooled non-essential amino acids could be utilized. This experimental approach has provided comparative cell cultures for studying free amino acid pools which are minimally influenced by population development.

The results show that the free amino acid pool in L cells is freely expandable for all of the amino acids. The internal level for any amino acid is characteristically determined by the concentration of the medium(6). The extent of concentration of any single amino acid also appears to be dependent upon the other amino acids in the media. In general, non-essential amino acids are more highly concentrated than essential amino acids when every amino acid is present in culture media at a similar level. When non-essential amino acids are omitted from the medium, they are even more effectively concentrated. A concentration ratio of 150 has been noted for glycine when it is not included in the tissue culture medium(7). In this situation the non-essential amino acids must be synthesized endogenously; however, this does not mean that an impermeable barrier is obstructing the free exchange of these endogenously synthesized amino acids with the extracellular environment. It is well established that amino acids leak from the intracellular environment of mammalian cells. Such amino acids must be reconcentrated by the cells by active transport, a mechanism which appears to function more efficiently when substrates are present at low exogenous levels(8,9). A similar situation exists for potassium ion uptake by a variety of animal cells. It has been suggested that potassium concentration may be linked to amino acid transport(10).

The internal level of each essential amino acid is also determined by its respective external concentration, but if an essential amino acid is deleted from the culture medium, the pool becomes depleted of this amino acid; cell growth ceases(7). On the other hand, our results demonstrate that intracellular essential amino acid levels can be doubled in some cases by eliminating non-essential amino acids from the medium. At first, we thought this might occur because we had

been comparing populations with low and high concentrations of essential amino acids, and concentrative uptake would be favored in the medium with the lower levels of essential amino acids. However, the results show that the intracellular levels for all of the essential amino acids are higher when the essential amino acid levels in MEMS are raised to those concentrations present in 199P. This can only indicate that some exclusion of amino acids is occurring in medium 199P, perhaps by other amino acids. Glycine and alanine have been shown to inhibit competitively the uptake of α-amino isobutyric acid in L cell cultures(9).

The differences observed in the accumulation of amino acids in our experiments seem to imply that a membrane discrimination step occurs. The pooling of non-essential amino acids is favored in the L cell system. Removal of these amino acids from the growth medium enhances the accumulation of the essential amino acids. The selective pooling of non-essential amino acids resembles the pattern of amino acid elution from a polyelectrolyte as we have previously noted. It is interesting that the non-essential amino acids which are highly concentrated by L cells are all eluted through a specific range from the cation exchange resin column used in our experiments, while the essential amino acids are eluted through another range. It is striking that threonine which is an essential amino acid is eluted from the resin column with the non-essential amino acids, and it is concentrated like a non-essential amino acid.

Summary. Replacement of 199P medium which contains 21 amino acids with a basal medium containing only the minimum essential amino acids does not alter the development of a cell population *in vitro,* but does modify the nature of the free amino acid pool. The non-essential amino acids constitute about 80% of the intracellular pool when the cells are grown in 199P, and most of these amino acids are concentrated by the L cells. In the modified media which contain only the essential amino acids, intracellular levels of the non-essential amino acids decrease while internal levels of the essential amino acids increase. However, the cells retain a selective capacity to concentrate the non-essential amino acids, since steeper gradients for these amino acids are noted between external and internal environments. Intracellular levels of any essential amino acid can be increased by raising the level in the culture medium, but this too does not influence the development of the cell population.

1. Eagle, H., Piez, K. A., Levy, M., *J. Biol. Chem.,* 1961, v236, 2039.

2. Kuchler. R. J., Grauer, R. C., *Biochim. Biophys. Acta,* 1962, v57, 534.

3. Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.,* 1958, v30, 1190.

4. Salzman, N. P., Eagle, H., Sebring, E. D., *J. Biol. Chem.,* 1958, v230, 1001.

5. Campbell, A., *Bact. Rev.,* 1957, v21, 263.

6. Cowie, D., McClure, F. T., *Biochim. Biophys. Acta.,* 1959, v31, 236.

7. Piez, K. A., Eagle, H., *J. Biol. Chem.,* 1958, v231, 533.

8. Heinz, E., *ibid.,* 1957, v228, 97.

9. Maio, J. J., Rickenberg, H. V., *Exp. Cell Res.,* 1962, v27, 31.

10. Christensen, H. N., Riggs, T. R., Coyne, B. A., *J. Biol. Chem.,* 1954, v209, 413.

Received September 3, 1963. P.S.E.B.M., 1963, v116.