THE UTILIZATION OF PHENYLALANINE AND TYROSINE FOR PROTEIN SYNTHESIS BY HUMAN CELLS IN TISSUE CULTURE

BY HARRY EAGLE,* K. A. PIEZ,† AND RALPH FLEISCHMAN*

(From the National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland)

(Received for publication, March 20, 1957)

The amino acid requirements of a mouse fibroblast and of a number of normal and malignant human cells in tissue culture have been described in previous communications (1-4). All the cell lines examined, whether normal or malignant, required thirteen amino acids for survival and growth (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine). The methods used for the determination of the nutritionally essential amino acids permitted a direct approach to their metabolic utilization. The present paper describes the incorporation into protein and metabolic utilization of phenylalanine and tyrosine by three human cell lines, two deriving from normal liver and conjunctiva, and one from a carcinoma of the cervix. The data also provide evidence with respect to protein turnover in these cultures.

EXPERIMENTAL

Growth and Preparation of Cell Suspension—The three cell lines used in these studies were strain HeLa (5), and two cultures deriving from normal human liver and conjunctiva and originally isolated by Chang (6). Although these two cells are hereafter referred to simply as “liver” and “conjunctiva,” this is not meant to imply that the cells are in fact hepatic or conjunctival epithelium or that they have the same biochemical activities as the corresponding cells in the intact host.

The basal growth medium used in these experiments embodied (a) the thirteen amino acids previously shown to be essential for all cell strains so far studied (1-4); (b) the eight essential vitamins (7-9) (choline, folic acid, myo-inositol, nicotinamide, pantothenic acid, pyridoxal, thiamine, and riboflavin); (c) salts (10); (d) glucose; (e) serum protein, the last added as 5 per cent dialyzed human serum. The concentrations of the various components of the medium have recently been summarized (8).

* Section on Experimental Therapeutics, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases.
† National Institute of Dental Research.
For the present experiments, requiring relatively large amounts of material, the cells were grown as a layer adherent to the bottom of 1 liter Blake bottles, overlaid with 60 to 85 ml. of medium, and inoculated with approximately 5 million cells. In the experiments which involved growing cell cultures, the complete medium, containing randomly C¹⁴-labeled phenylalanine or tyrosine (Schwarz Laboratories, Inc.), was added to a young culture in the stage of active proliferation. In such cultures, the amount of cells (measured in terms of either volume, dry weight, protein, total nitrogen, or protein nitrogen) increased by 40 to 100 per cent in 24 hours and by 75 to 250 per cent in 48 hours. Although the amount of growth varied in individual experiments, the several parameters of growth gave quantitatively consistent results.

To study the incorporation and utilization of phenylalanine and tyrosine when the cells were not growing, one or more of the other essential amino acids were omitted from the medium. Under these conditions there was no net growth, and the cell mass and protein either remained unchanged or gradually decreased over the 24 to 72 hour period of observation (Table I). In most of the experiments carried out with such deficient media, the cells were incubated for 12 hours in an unlabeled but similarly deficient medium, before the addition of fresh medium containing the randomly C¹⁴-labeled phenylalanine or tyrosine. However, that preliminary depletion had no demonstrable effect on the subsequent incorporation of the labeled amino acids into protein.

When the cells were ready for harvesting, the supernatant fluid was withdrawn, and the adherent cells were washed twice with the balanced salt solution. In most of the experiments, the cells were then scraped off the glass with a rubber policeman, collected in 5 to 10 ml. of fluid, centrifuged in a Shevsky-Stafford tube, and the measured sediment was frozen until ready for use. It was then emulsified in 19 volumes of water, and the cells were disintegrated by sonic oscillation for 15 minutes in a 9 kc. Raytheon sonic oscillator. In later experiments, the intact cell layer on the surface of the bottle was washed with cold salt solution, and then treated directly with cold 8 per cent trichloroacetic acid (TCA) (10 to 20 ml. per bottle), without preliminary scraping. The coagulated cell mass was then harvested, emulsified in a Potter-Elvehjem glass grinder, centrifuged, and washed with cold 8 per cent TCA. The latter method yielded as much as 30 per cent more protein N than did the scraping procedure. This reflects the vulnerability of the cells to manipulation before treatment with TCA.

Depending upon the size of the inoculum, the type of medium, and the duration of incubation, from 50 to 200 mg. wet weight of cells were obtained from each Blake bottle; the cells from three to twenty bottles were combined for each determination. Similar harvests of three to six bottles
were prepared as controls at the time the labeled medium was added to the cell.

The TCA precipitate was extracted successively with 95 per cent alcohol and ether, and was then extracted twice with 5 per cent hot TCA (3 ml. per ml. of sonate) at 90° for 15 minutes. The centrifuged "protein" residue was again extracted with alcohol and ether, and then hydrolyzed in

Table I
Illustration of Absence of Net Protein Synthesis in Cell Cultures Deprived of One or More Nutritionally Essential Amino Acids

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Amino acids omitted from growth medium</th>
<th>Time of incubation (hrs.)</th>
<th>Dry weight (mg.)</th>
<th>Total N (mg.)</th>
<th>Protein N (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Tyrosine</td>
<td>0</td>
<td>142</td>
<td>16.5</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>136</td>
<td>13.0</td>
<td>8.62</td>
</tr>
<tr>
<td></td>
<td>Arginine, histidine, lysine, tyrosine, tryptophan</td>
<td>0</td>
<td>96.2</td>
<td>10.6</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>83.9</td>
<td>9.11</td>
<td>5.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>79.1</td>
<td>8.94</td>
<td>5.58</td>
</tr>
<tr>
<td></td>
<td>Cystine, isoleucine, leucine, methionine, threonine, valine</td>
<td>0</td>
<td>94.6</td>
<td>9.64</td>
<td>7.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>81.8</td>
<td>8.18</td>
<td>6.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>79.4</td>
<td>8.67</td>
<td>6.27</td>
</tr>
<tr>
<td>Liver</td>
<td>Tyrosine</td>
<td>0</td>
<td>76.4</td>
<td>8.42</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>72.9</td>
<td>7.78</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>Cystine, isoleucine, leucine, methionine, threonine, tyrosine, valine</td>
<td>0</td>
<td>76.4</td>
<td>8.42</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>79.6</td>
<td>8.22</td>
<td>5.85</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>Cystine, isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine, valine</td>
<td>0</td>
<td>99.0</td>
<td>9.7</td>
<td>6.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>94.5</td>
<td>8.93</td>
<td>6.26</td>
</tr>
</tbody>
</table>

6 N HCl in a sealed tube at 121° (15 pounds autoclave pressure) for 18 hours. In some experiments the sonically disintegrated cells were extracted directly with hot TCA. The two procedures gave similar values for cell "protein."

Determination of Tyrosine and Phenylalanine—The ion exchange methods were patterned after those described by Moore and Stein (11, 12) and modified for these experiments, as previously described by us (13). For the determination of protein tyrosine and phenylalanine alone, the hydrolysate was evaporated several times to a syrup, made up to a convenient volume, and an aliquot containing 2 to 3 mg. of N placed on the top of a 100 × 0.9
cm. column of Dowex 50-X12, minus 400 mesh, jacketed at 50°. It was washed in with two small portions of buffer, pH 5.00 (6.8 gm. of citric acid monohydrate and 20 gm. of sodium citrate dihydrate per liter) containing 1 ml. of BRIJ 35 solution (cf. Moore and Stein (11)) per 100 ml. The column was eluted with the same buffer at 8 ml. per hour. 1 ml. fractions were collected, diluted with 2 ml. of water, and 1 ml. aliquots from Tubes 76 through 125 were analyzed with ninhydrin (14). The remainder was saved for measurement of C¹⁴ activity. The column was washed with approximately 50 ml. of 0.2 N NaOH after each run and then equilibrated with buffer, pH 5.00.

In some experiments a complete amino acid analysis was performed to provide data for other purposes. In these cases the procedure previously described (13) was used, except that 1 ml. fractions were collected and diluted with 2 ml. of water. Fig. 3 shows an example of the tyrosine and phenylalanine portions of such a complete run. Both methods gave approximately the same resolution of tyrosine and phenylalanine.

Measurement of Radioactivity—Determinations of radioactivity were made in a gas flow counter. 0.5 ml. samples of varying dilutions were dried in duplicate in 1 inch copper planchtets, and a second pair of plates was prepared containing the same amount of unknown solution plus a known amount of the C¹⁴-labeled precursor amino acid which had been added to the medium. A simple calculation gave the C¹⁴ content of the unknown solution relative to that of the amino acid added to the medium, automatically corrected for self-absorption.

\[
C^{14} \text{ sample} = C^{14} \text{ standard} \times \frac{\text{counts (sample)}}{\text{counts (sample + standard)} - \text{counts (sample)}}
\]

Results

Incorporation of C¹⁴-Phenylalanine and C¹⁴-Tyrosine into Protein by Growing Cells—As shown in Table II, when uniformly C¹⁴ labeled phenylalanine or tyrosine was incorporated by any of the three cell lines from a complete growth medium containing the labeled amino acid at the optimal growth concentration of 0.1 mM (2), 78 to 97 per cent of the C¹⁴ incorporated by the cells appeared in the cell protein. Significant amounts, usually on the order of 4 per cent of the total, were further recovered as phenylalanine (or tyrosine) in the cold TCA extract of the cells,1 traces only were found in the hot TCA extract, and varying amounts, usually on the order of 5 to 10 per cent of the total incorporated, appeared in the alcohol-ether extracts of the cold TCA residue. Of the protein C¹⁴, 79 to 92 per cent was re-

1 The amount of amino acid extracted by cold TCA varied widely according to whether it was added to the intact cell layer or to the sonically disintegrated cell suspension (Piez, K. A., and Eagle, H., in preparation).
covered as the parent amino acid, and, except for the conversion of phenylalanine to tyrosine, as discussed in a following section, no other amino acid was significantly labeled. Under the conditions of the present experiments, most of the phenylalanine or tyrosine taken up by the cells was incorporated into protein as the specific amino acid, with but little conversion to other cellular components.

Fig. 1 summarizes the results obtained in a number of experiments in which the amount of C\textsuperscript{14}-phenylalanine incorporated into protein was determined after 24 or 48 hours growth in a complete medium containing the labeled amino acid at 0.1 mM. As indicated by the open points in Fig. 1, in all the experiments, and with all three cell lines, more C\textsuperscript{14}-labeled phenyl-

![Table II](image)

<table>
<thead>
<tr>
<th>Labeled amino acid added to medium</th>
<th>Cell strain</th>
<th>Per cent of cell C\textsuperscript{14} in protein</th>
<th>Per cent of protein C\textsuperscript{14} in phenylalanine (tyrosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>HeLa</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Conjunctiva</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>HeLa</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Conjunctiva</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>86</td>
<td>84</td>
</tr>
</tbody>
</table>

alanine was incorporated than could be accounted for according to the amount of cell growth and new protein formed. The less the amount of growth relative to the initial cell mass, the greater was the magnitude of this discrepancy.

Incorporation of Phenylalanine by Non-Growing Cells—The probable explanation of the discrepancy came with the finding that C\textsuperscript{14}-labeled phenylalanine or tyrosine was incorporated into cell protein even when growth had been prevented by the omission from the medium of one or more essential amino acids. This is shown in the experiments of Tables III and IV. In every experiment, despite the absence of net protein synthesis, the labeled amino acid was nevertheless incorporated into protein, again largely as phenylalanine or tyrosine itself, and, except for glutamine, it was incorporated to the same degree whether one, two, or seven amino acids had been omitted from the medium. Further, as shown in the experi-
ments with the HeLa cell (Table III), this incorporation in the absence of growth proceeded at a constant rate over a 72 hour period.

**Fig. 1.** The discrepancy between the amounts of C\(^{14}\)-phenylalanine or C\(^{14}\)-tyrosine incorporated into cell protein by growing cultures, and the amount of new protein synthesized. HeLa cell, ○, phenylalanine, △, tyrosine; liver, □, phenylalanine, ▽, tyrosine; conjunctiva, ◊, phenylalanine, ▽, tyrosine. Open circles represent total C\(^{14}\)-amino acid incorporated, exceeding the absolute increase in corresponding amino acid residues of protein. Solid circles indicate net incorporation associated with growth, after subtraction for C\(^{14}\)-amino acid incorporated into initial cell protein (assuming the same 1 per cent per hour rate as in non-growing cultures).

The results in three experiments with HeLa cells, in which the amount of phenylalanine incorporated into protein was determined at several time periods, are summarized in Fig. 2. As shown, the incorporation proceeded
like a first order reaction, in that the log (residual unlabeled phenylalanine/total phenylalanine) decreased linearly with time, with a slope corresponding to an incorporation rate \((k)\) of approximately 1 per cent per hour. The values for \(k\) for the HeLa cell in a number of experiments are listed in Table III, last column, and averaged approximately 1 per cent per hour. The results obtained in similar experiments with both amino acids and all three cell lines are summarized in Table IV. As shown, protein amino acid residues were replaced by labeled amino acid from the medium at a uniform rate, varying between 0.74 and 1.1 per cent per hour.

The excessive incorporation of \(^{14}\text{C}-\text{phenylalanine}\) or \(^{14}\text{C}-\text{tyrosine}\) by growing cells (cf. Fig. 1) was apparently due to a similar incorporation into the protein of the initial cell population, over and above the incorporation associated with the synthesis of new protein. If the total \(^{14}\text{C}-\text{phenylalanine}\) or \(^{14}\text{C}-\text{tyrosine}\) uptake into the protein of growing cells was corrected for this factor, assuming a replacement rate of 1 per cent per hour, the resulting net incorporation associated with growth then agreed reasonably well with the actual phenylalanine content of the newly synthesized protein (cf. Fig. 1).

**Role of Glucose and Glutamine in Non-Growth Incorporation of Phenylala-**
PHENYLALANINE AND TYROSINE

nine into HeLa Cell Protein—It is of interest that the incorporation of labeled phenylalanine by non-growing HeLa cells was relatively unaffected by the omission of glucose from the medium, but was approximately halved by the omission of glutamine (Table V). The omission of both glucose and glutamine had only slightly more effect than the omission of glutamine alone, and the further omission of other essential amino acids did not affect the rate of incorporation.

Conversion of Phenylalanine to Tyrosine by Variant of HeLa Cell—The results obtained with an atypical HeLa strain differed in one important

---

**Table III**

Incorporation of $^{14}$C-Labeled Phenylalanine by HeLa Cells in Absence of Growth

Cells were overlaid for the indicated period of time with a medium deficient in one to seven amino acids, but containing randomly $^{14}$C-labeled phenylalanine. The horizontal rows represent experiments carried out at different times, with varying cell populations, and the amounts of phenylalanine incorporated are therefore not comparable. The amount of labeled phenylalanine incorporated into protein was independent of the number of amino acids omitted from the medium, and the several types of experiment are not distinguished below.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Specific activity of protein phenylalanine*</th>
<th>Rate of phenylalanine replacement</th>
<th>Average ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td></td>
<td>per cent per hr.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.152</td>
<td>1.4</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>0.117</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.177</td>
<td>0.81</td>
<td>1.02 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.206</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.179</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.232</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.204</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.289</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.236</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.309</td>
<td>0.78</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.306</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.300</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.446</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.467</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.200</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.344</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>0.376</td>
<td>0.66</td>
<td>0.91 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.545</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Total average</td>
<td></td>
<td></td>
<td>1.0 ± 0.05</td>
</tr>
</tbody>
</table>

* Referred to initial activity of phenylalanine in medium as 1.
TABLE IV

Incorporation of C\textsuperscript{14}-Tyrosine and C\textsuperscript{14}-Phenylalanine by Human Cells in Tissue Culture in Absence of Net Protein Synthesis

Growth was prevented by the omission of one to seven essential amino acids from the medium. The concentration of tyrosine (phenylalanine) was 0.1 mM throughout, and the time of incubation 48 hours.

<table>
<thead>
<tr>
<th>C\textsuperscript{14}-labeled amino acid in medium</th>
<th>Cell strain</th>
<th>μmoles of C\textsuperscript{14}-amino acid incorporated by cells</th>
<th>Specific activity of protein tyrosine (phenylalanine)*</th>
<th>Rate at which initial protein tyrosine (phenylalanine) was replaced by C\textsuperscript{14}-amino acid from medium per cent per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>HeLa</td>
<td>4.17 3.21 2.73 0.30 0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td></td>
<td>4.13 3.04 2.37 0.31 0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctiva</td>
<td></td>
<td>3.1† 2.4 2.12 0.23† 1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.2 11.5 9.81 0.38 0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Liver</td>
<td>4.28 3.28 2.68 0.30 0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.67 3.48 2.79 0.34 0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conjunctiva</td>
<td>4.89 4.16 3.12 0.291 0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.02 2.6 2.64 0.338 0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.2 3.31 2.99 0.386 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.64 4.0 3.5 0.382 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Referred to initial activity of C\textsuperscript{14}-phenylalanine or C\textsuperscript{14}-tyrosine in medium as 1.
† 24 hour experiment.

Fig. 3. A portion of the effluent from the ion exchange analysis of hydrolyzed protein from a variant HeLa strain, showing the conversion of phenylalanine to tyrosine. The cells were supplied with C\textsuperscript{14}-phenylalanine and deprived of tyrosine. ●, amino acid concentration (μmoles per ml.); ○, C\textsuperscript{14} activity (counts per minute per ml.).
PHENYLALANINE AND TYROSINE

respect from those described above. This variant appeared spontaneously in a stock culture, and was first evidenced by a decreased growth rate and by a change in the microscopic appearance of the cells. These now grew out as discrete, spindle-shaped cells which resembled fibroblasts, rather than the mosaic sheet of epithelium characteristic of the parent HeLa strain, still present in parallel stock cultures. In a complete growth medium this variant strain behaved like the parent culture, in that essentially all of the C14-phenylalanine incorporated into protein was identified as phenylalanine itself, and there was no demonstrable conversion to tyrosine. However, when tyrosine was omitted from the medium, there was significant conversion of phenylalanine to tyrosine, and labeled tyrosine appeared in the protein fraction (cf. Table VI and Fig. 3). This conversion of phenylalanine to tyrosine was observed whether tyrosine was the only amino acid missing from the medium or whether a number of other amino acids were simultaneously omitted. Conversely, the presence of tyrosine completely suppressed its formation from phenylalanine, whether in a complete growth medium or in a medium lacking other essential amino acids.

With this HeLa variant, 12 to 25 per cent of the total C14-phenylalanine incorporated into protein in a tyrosine-deficient medium was present as

<table>
<thead>
<tr>
<th>Essential growth factors omitted from medium</th>
<th>Time of incubation</th>
<th>Specific activity of protein phenylalanine*</th>
<th>Rate of replacement of protein phenylalanine by C14-phenylalanine from medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>hrs.</td>
<td>per cent per hr.</td>
<td></td>
</tr>
<tr>
<td>+ 6 amino acids</td>
<td>24</td>
<td>0.28</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.20</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.18</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.17</td>
<td>0.78</td>
</tr>
<tr>
<td>+ glucose</td>
<td>24</td>
<td>0.12</td>
<td>0.53</td>
</tr>
<tr>
<td>+ 6 other amino acids</td>
<td>24</td>
<td>0.1</td>
<td>0.44</td>
</tr>
<tr>
<td>+ glucose</td>
<td>24</td>
<td>0.079</td>
<td>0.34</td>
</tr>
<tr>
<td>+ 6 other amino acids</td>
<td>24</td>
<td>0.091</td>
<td>0.40</td>
</tr>
<tr>
<td>+ glucose</td>
<td>24</td>
<td>0.098</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.086</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.145</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.23</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* Referred to initial activity of phenylalanine in medium as 1.
tyrosine. The specific activity of the protein tyrosine varied in individual experiments, and was 20 to 50 per cent that of the protein phenylalanine itself. Between them, these two amino acids accounted for 92 to 96 per cent of the total C\(^{14}\) incorporated into protein. Under the conditions of these experiments, phenylalanine was thus specifically converted to tyrosine, and there was no significant conversion to other amino acids.

In the first experiment of Table VI, the amount of tyrosine formed from phenylalanine by this atypical HeLa strain and incorporated into protein was 0.3 \(\mu\)mole per gm. of cell per hour, equivalent to approximately 0.4 per cent of the total protein tyrosine per hour. Despite this significant rate of tyrosine synthesis, the cell strain proved incapable of sustained growth in a tyrosine-deficient medium.\(^2\)

**DISCUSSION**

Under the conditions of the present experiments, the primary metabolic function of phenylalanine and tyrosine appeared to be their direct incorporation into protein, with no significant conversion to other amino acids. An exception was provided by a variant HeLa culture which proved capable...

\(^2\) On prolonged cultivation, this variant strain has since lost its ability to form tyrosine from phenylalanine, with no further gross change in its morphologic appearance.
ble of oxidizing phenylalanine to tyrosine. The amount of C$^{14}$-labeled tyrosine so formed and incorporated into protein represented the replacement of as much as 0.4 per cent of the initial protein tyrosine per hour with newly synthesized amino acid. Despite this continuing production of tyrosine, the cells proved incapable of growing in a tyrosine-deficient medium, and eventually died.

The hydroxylation of phenylalanine by this HeLa variant differs from the reaction described by Udenfriend and Cooper (15) in liver tissue slices$^3$ in that it was completely inhibited by the presence of tyrosine in the growth medium. In the case of the liver hydroxylase, a 17-fold excess of tyrosine over phenylalanine had no effect on the course of the reaction.

In a growing culture, the amount of phenylalanine or tyrosine incorporated into protein by all three cell lines tested regularly exceeded that anticipated from the amounts of new protein formed. The probable explanation was the observation that, as has been regularly observed in experiments with tissue slices or cell suspensions, these amino acids were incorporated into protein in the absence of net synthesis. A similar incorporation of labeled amino acid into the initial cell protein apparently took place even under conditions of growth. When the total amino acid incorporated was corrected for this factor, the net incorporation then agreed with that anticipated in view of the amounts of new protein formed.

Approximately 0.7 to 1 per cent of the total phenylalanine (tyrosine) residues of the cell protein of non-growing cultures exchanged with labeled amino acids in the medium per hour. This rate was several times greater than has been reported for tissue slices (16), for cultures of tissue explants in plasma clots (17), or for cell particulate fractions (18). It was, however, of the same order of magnitude as the initial rate of incorporation in short term experiments with cancer cell suspensions (19, 20). The unusual aspect of the present experiments was the fact that this incorporation continued at an unchanged rate for a period of 72 hours, by which time more than 50 per cent of the total phenylalanine residues of the protein had been replaced with labeled amino acid from the medium.

In explanation of the fact that labeled phenylalanine and tyrosine were incorporated into cell protein in a nutritionally deficient medium in which there was no net protein synthesis, the same possibilities must be considered which enter into any discussion of protein turnover.

$^3$ It is to be noted that the "liver" culture used in these experiments failed to hydroxylate phenylalanine. One can only speculate (a) whether a culture of liver epithelium had lost some of its specialized biochemical functions upon prolonged cell cultivation in vitro; (b) whether the full biochemical potential of the cell is not demonstrable under the conditions of cultivation in vitro here used; or (c) whether the cell which originally grew out from the liver explant was not hepatic epithelium, but some other cell type.
(a) As in the exchange reaction described by Gale and Folkes in the case of bacteria (21), the labeled phenylalanine or tyrosine of the medium may enter the amino acid pool of the cell, and then exchange with unlabeled amino acid residues in an otherwise intact protein molecule, and without its preliminary degradation.

(b) In media deficient in one or more essential amino acids, some cells are dying. The cell products thereby released may be used for the synthesis of new cells, the amount of that synthesis being limited by the amounts of the missing amino acids thereby made available. The specific activity of the protein phenylalanine (tyrosine) in the new cells formed would then be essentially that of the corresponding labeled amino acid in the medium.

(c) In the absence of net synthesis or cell turnover, the cells may nevertheless be releasing protein into the medium, which is then broken down in the medium and the products used for the synthesis of new protein as in (b).

(d) There may be active intracellular turnover of protein, as suggested by Schoenheimer (22) and more recently indicated by a number of studies with normal and malignant cells and tissues (23–28). Such protein turnover within mammalian cells would contrast with the situation in bacteria, in which several classic experiments indicate that the formed proteins of the cell are not utilized for the de novo biosynthesis of enzymes (29, 30). Experiments bearing on these several possibilities are now in progress.

It is of particular interest that the rate of incorporation of labeled phenylalanine and tyrosine by non-growing cells was not demonstrably affected by the omission of glucose from the medium. This contrasts with the finding of many workers (19, 20, 23, 28) that the incorporation of amino acids into cellular proteins depends upon an exogenous energy source, usually supplied as glucose. In the present experiments, the medium contained relatively large amounts of glutamine. This has been shown to be rapidly converted by tissue culture cells to glutamic acid, aspartic acid, and proline, and to a lesser degree to glycine, alanine, and serine as well (31); its catabolism might conceivably have provided the necessary energy for incorporation. However, upon the omission of glutamine from the medium, the incorporation of phenylalanine or tyrosine into protein was approximately halved, to 0.3 to 0.5 per cent per hour, but remained at this significant level even when glucose and all other amino acids were also omitted from the medium, leaving only vitamins, phenylalanine, and salts. One must conclude that the incorporation here observed is not energy-dependent, unless that energy derives from the catabolism of phenylalanine or of some of the cell components. The reduced incorporation caused by the omission of glutamine could depend upon an entirely different factor.
These tissue culture cells have been shown to break down glutamine to glutamic acid and ammonia (31). In the absence of a glutamine reservoir in the medium, the deamidating system would compete with the protein-synthesizing system for the relatively small amounts of glutamine liberated either by cell turnover, by protein secretion, or by the intracellular turnover of protein (hypotheses (b), (c), (d) above). In any case, the amount of protein resynthesis would be decreased to the degree that the glutamine was in fact hydrolyzed.

SUMMARY

The metabolic utilization of uniformly C\textsuperscript{14}-labeled phenylalanine and tyrosine by three human cell strains in tissue culture (uterine carcinoma (HeLa), normal liver, and normal conjunctiva) has been examined with the following findings:

1. With both amino acids and all three cell lines, the C\textsuperscript{14} was incorporated almost entirely into protein, and as the corresponding amino acid.

2. The one exception was a variant strain of HeLa which was able to convert phenylalanine to tyrosine, though not at a rate sufficient to support growth. This hydroxylase activity was manifested only in the absence of tyrosine.

3. Both amino acids were rapidly incorporated into cell protein in the absence of net protein synthesis, from media lacking one or more essential amino acids other than glutamine. With all three cell lines, 0.7 to 1 per cent of the phenylalanine or tyrosine residues of the protein was replaced by the labeled amino acid of the medium per hour. This rate was sustained for 3 days, and was approximately halved by the omission of glutamine from the medium.

4. In growing cultures, more phenylalanine or tyrosine was incorporated into cell protein than could be accounted for by the amount of growth. The excess was consistent with the thesis that the amino acid residues in the initial cell protein were being replaced by labeled amino acid from the medium at approximately the same rate as in non-growing cultures.

5. Possible mechanisms of this incorporation, not associated with growth, are discussed.

The assistance of Mrs. Louise M. Morris in the conduct of these experiments is gratefully acknowledged.

BIBLIOGRAPHY
