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Metabolic Properties of the Products of Mitochondrial Protein Synthesis in HeLa Cells*

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The metabolic behavior of the mitochondrial protein synthesis products has been investigated in HeLa cells. Particular attention was given to the four major electrophoretic components (designated as Nos. 2, 3, 5, and 8) of the 10 previously identified as organelle-specific products.

Inhibition of cytoplasmic protein synthesis with emetine or cycloheximide causes a rapid decline in the rate of mitochondrial protein synthesis, with an estimated half-life of 1 to 2 h, affecting in a parallel way all the discrete components. About 30% of the original synthetic activity appears to be resistant to emetine treatment for at least 24 h; however, all the polypeptides synthesized after the first 4 h of cell exposure to emetine are metabolically unstable, possibly because of lack of integration into the inner mitochondrial membrane.

An analysis of the stability of newly synthesized products of mitochondrial protein synthesis pulse-labeled in the presence of cycloheximide and then chased in the absence of the drug (*i.e.* under conditions of resumed cytoplasmic protein synthesis) has revealed marked differences among the various discrete components. In particular, about three-fourths of the radioactivity associated with components 3 and 5 decays within 4 h of chase, the remainder being substantially stable afterwards; by contrast, the radioactivity in components 2 and 8 shows only a slow decline during a 3-day chase. If the chase is carried out under conditions of a persistent block of cytoplasmic protein synthesis, as is the situation after a pulse labeling in the presence of emetine, all newly synthesized components appear to be destabilized in various degrees, with the exception of component 5, which is to a great extent stabilized.

Inhibition of mitochondrial protein synthesis with chloramphenicol has a progressive stabilizing effect on most of the discrete components newly synthesized after removal of the drug; this effect is especially striking in the case of component 5 which, in experiments of continuous labeling in the presence of emetine after prolonged chloramphenicol treatment, becomes, after 24 h of labeling or more, the only recognizable peak in the electrophoretic pattern of the sodium dodecyl sulfate-lysed mitochondrial fraction.

The results of the kinetic experiments described here are interpreted in terms of two roles of cytoplasmically synthesized proteins, one required for the synthesis of polypeptides within the organelles, the other necessary for the stabilization of the mitochondrial products.

In spite of the large amount of work which has been carried out on mitochondrial protein synthesis both in lower and higher eukaryotic cells (see reviews by Ashwell and Work (1) and Beattie (2)), very little is known about the metabolic properties of the products of this synthesis. Previous investigations have pointed to the role of cytoplasmically synthesized proteins in controlling the rate of mitochondrial protein synthesis or the integration into the inner membrane of its products (see review by Schatz and Mason (3)). However, they have provided no information concerning the metabolic stability of the mitochondrial products under physiological conditions or in the absence of cytoplasmic protein synthesis.

Earlier work from this laboratory has led to the identification, among the *in vivo* products of mitochondrial protein synthesis in HeLa cells, of 10 distinct electrophoretic components in the molecular weight range from about 11,000 to 42,000 (4).

In this work, inhibition of cytoplasmic protein synthesis by emetine or cycloheximide was found to cause a rapid decline in the rate of mitochondrial protein synthesis, affecting in a parallel way all the discrete components. Marked differences between these components were observed when the metabolic stability of the newly synthesized polypeptides was examined under conditions of cytoplasmic protein synthesis. Furthermore, the stability of these components was differentially affected under conditions of inhibition of cytoplasmic protein synthesis or after a prolonged block of mitochondrial protein synthesis. In particular, component 5 (M_r = about 27,500) was found to be preferentially stabilized over the other components. These results point to a complex and finely modulated interplay between cytoplasmic and mitochondrial protein synthesis, with cytoplasmic protein products playing an essential

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role both for the synthesis and the stabilization of the mitochondrially synthesized polypeptides.

EXPERIMENTAL PROCEDURES

Growth of Cells—Suspension cultures of HeLa cells were grown as previously described (5). The cultures were free of any detectable *Mycoplasma* contamination.

Labeling Conditions—*In vivo* pulse labeling of the products of mitochondrial protein synthesis in HeLa cells was carried out as previously described (4), except that the suspensions to be labeled had a cell concentration of up to 3×10^6/ml. For details about experimental procedures concerning cell exposure to antibiotics, long term labeling, and chase experiments, see the legends to the figures.

Subcellular Fractionation and Disruption of Mitochondrial Membranes—Reference is made to previous reports for the preparation of the $5000 \times g$ crude mitochondrial fraction (6, 7) and its disruption by sonication (4).

Polyacrylamide Gel Electrophoresis—The disrupted mitochondria were dissolved and electrophoresed as described previously (4), except that the samples were not heated before electrophoresis. The samples applied to the gels contained 120 to 600 μg of protein.

RESULTS

Effects of Pretreatment with Cycloheximide or Emetine on Growth of HeLa Cells—Two inhibitors of cytoplasmic protein synthesis, cycloheximide (7–10) and emetine (11–13), were utilized in the present work in order to specifically label the products of mitochondrial protein synthesis. Fig. 1 shows the growth curves of cells exposed to cycloheximide for 30 min (*Panel a*), 4 h or 8 h (*Panel b*), or to emetine for 30 min (*Panel c*), and then resuspended in fresh, drug-free medium. Cells treated with cycloheximide for 30 min or 4 h exhibited a growth curve virtually indistinguishable from that of control cells, indicating a complete reversibility of the effects of this drug, in agreement with previous findings (8, 15). After 8 h of cell exposure to cycloheximide, there was a beginning of cell death, with the majority of the cells (>85%), however, surviving and starting to grow at a normal rate, possibly after a short lag. By contrast, cells treated with emetine for only 30 min did not resume growth after removal of the drug from the medium and, eventually, started disintegrating; this effect was presumably due to the irreversible action of emetine on protein synthesis (11).

In the present work, cycloheximide was used in the experiments where a reversibility of the block of cytoplasmic protein synthesis was required; in all the other cases, emetine was preferred because of the more complete inhibition of cytoplasmic protein synthesis (4, 12, 13).

Decay of Rate of Mitochondrial Protein Synthesis during Emetine or Cycloheximide Block of Cytoplasmic Protein Synthesis—The effect of a block of cytoplasmic protein synthesis on the rate of mitochondrial protein synthesis was first investigated. For this purpose, HeLa cells were labeled for 15 min with [^{3}H]isoleucine in the presence of emetine after different times of exposure to the antibiotic, ranging between 5 min and 24 h, and the trichloroacetic acid-precipitable radioactivity associated with the mitochondrial fraction was measured after each pulse (Fig. 2*a*, *filled circles, solid line*). It was previously shown that more than 90% of the radioactivity incorporated into the mitochondrial fraction, after a 60-min pulse of [^{3}H]isoleucine in the presence of 100 μg/ml of emetine, is chloramphenicol-sensitive (4). It appears from Fig. 2*a* that the rate of [^{3}H]isoleucine incorporation declines rapidly during the first 8 h of exposure to emetine, probably in an exponential fashion, with an estimated half-life of 1 to 2 h. After longer times of treatment with the drug, a relatively constant level of incorporation of [^{3}H]isoleucine, corresponding to about 30% of the level determined 5 min after addition of emetine, could be measured. A similar behavior was observed in another experiment, in which the [^{3}H]isoleucine incorporation sensitive to chloramphenicol (a specific inhibitor of mitochondrial protein synthesis (7, 9, 16)) in a 15-min pulse was measured, as a function of the length of emetine treatment of the cells, in total cell lysates (Fig. 2*a*, *dashed line*).

In order to test the possibility that the rapid initial decline observed in the rate of labeling with radioactive isoleucine of the mitochondrial protein products was due to emetine having a direct action on mitochondrial translation, as suggested by previous reports (17–19), the effect on mitochondrial protein synthesis of cycloheximide, an inhibitor which is apparently completely specific for cytoplasmic protein synthesis (17–19),

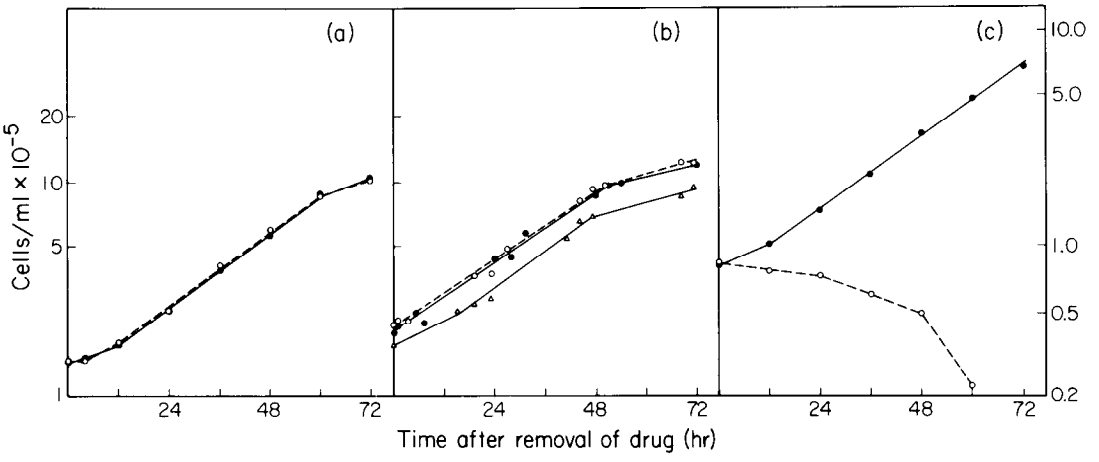

FIG. 1. Growth curves of HeLa cells after exposure to cycloheximide and emetine. HeLa cells (1 to 2×10^5 cells/ml) growing exponentially in modified Eagle's medium (14) were exposed to 100 μg/ml of cycloheximide for 30 min (*a*), 4 h, or 8 h (*b*). The cells were then washed once at 37° with cycloheximide-free medium and resuspended at the original concentration in the same medium; duplicate samples were counted at various intervals after the removal of the drug. Control cells were treated with buffer instead of cycloheximide. a: ○- - -○, cells treated with cycloheximide for 30 min; ●——●, control cells. *b*: ○- - -○, cells treated with cycloheximide for 4 h; △——△, cells treated with cycloheximide for 8 h; ●——●, control cells. *c*: conditions as described above, except for the initial concentration of the cells (~7×10^4 cells/ml) and the use of emetine (100 μg/ml) for 30 min instead of cycloheximide; ○- - -○, emetine-treated cells; ●——●, control cells.

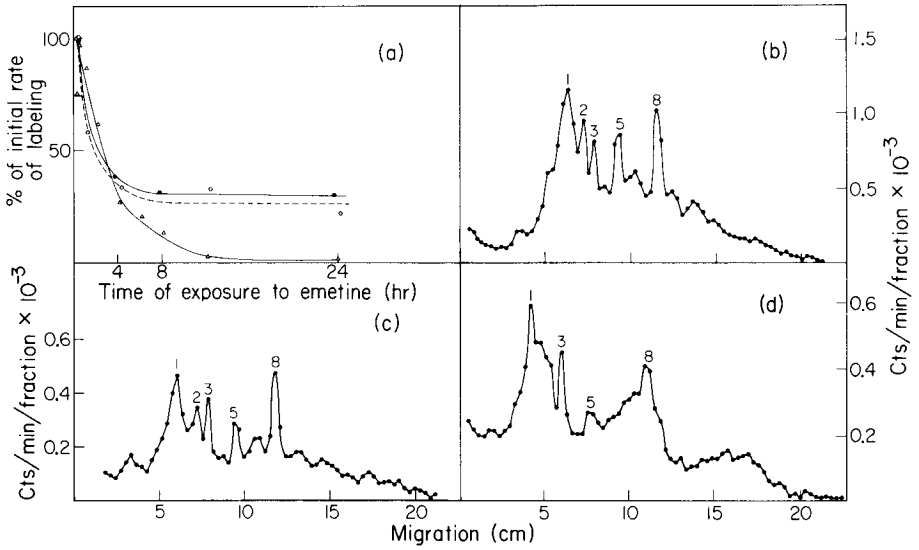

FIG. 2. *a*, kinetics of decline of the rate of mitochondrial protein synthesis in the presence of emetine or cycloheximide in control cells. HeLa cells (1.5×10^6/ml) were exposed to emetine (100 μg/ml) for various lengths of time and then labeled with 5 μCi/ml of [^{3}H]isoleucine for 15 min in isoleucine-free medium (4) in the presence of emetine and in the presence or absence of 100 μg/ml of chloramphenicol. The cells were then cooled, washed, resuspended in 0.25 M sucrose, 0.01 M Tris (pH 6.7 at 25°), 1.5×10^{-4} M $MgCl_2$ at 3 to 5 mg of protein/ml, sonicated as previously described (4), and lysed with 0.5% sodium dodecyl sulfate. A portion of each sample was precipitated with 15% trichloroacetic acid. In another experiment, cells were labeled for 30 min with [^{3}H]isoleucine in the presence of emetine after different times of exposure to the antibiotic, the mitochondrial fraction was prepared and sonicated, lysed with 0.5% sodium dodecyl sulfate, and precipitated with 15% trichloroacetic acid. In a third experiment, cells were labeled for 15 min with [^{3}H]isoleucine in the presence of 100 μg/ml of cycloheximide and in the presence or absence of 100 μg/ml of chloramphenicol, after different times of treatment with cycloheximide, and the mitochondrial fraction was prepared and analyzed as described above. ●——●, total acid-precipitable radioactivity associated with the mitochondrial fraction from cells treated with emetine (100% ≃ 8000 cpm (*cts/min*)/mg of protein); ○- - -○, chloramphenicol-sensitive acid-precipitable radioactivity associated with the whole lysate from cells treated with emetine (100% = 2390 cpm/mg of protein; chloramphenicol sensitivity = 41%); △——△, chloramphenicol-sensitive acid-precipitable radioactivity associated with the mitochondrial fraction from cells treated with cycloheximide (100% = 1315 cpm/mg of protein; chloramphenicol sensitivity = 44%). *b*, *c*, and *d*, electrophoretic profiles of the products of mitochondrial protein synthesis pulse-labeled with [^{3}H]isoleucine after 5 min, 4 h, or 12 h of exposure to emetine: 24-h long electrophoretic runs of the sodium dodecyl sulfate-lysed mitochondrial fractions from cells labeled with [^{3}H]isoleucine for 30 min after 5 min (*b*) or 4 h (*c*) of emetine treatment (corresponding to two of the time points of the experiment described in *Panel a* (●——●)), or for 1 h after 12 h exposure to the drug (*d*). The samples applied onto the gels contained about 600 μg of protein. The counts per min/fraction are normalized in all panels to 1 mg of protein of the mitochondrial fraction.

was investigated. As shown in Fig. 2*a* (*open triangles, solid line*), the rate of [^{3}H]isoleucine incorporation during a 15-min pulse declined exponentially to almost zero during the first 12 h of exposure of the cells to cycloheximide, with an estimated half-life of about 2 h. This result supports the idea that the decline of mitochondrial protein synthesis in the presence of emetine or cycloheximide is an indirect effect of the block of cytoplasmic protein synthesis.

Fig. 2, *b* and *c*, shows the electrophoretic profiles of the products of mitochondrial protein synthesis labeled with [^{3}H]isoleucine for 30 min after, respectively, 5 min and 4 h of exposure of the cells to emetine; the major peaks have been designated by progressive numbers in order of decreasing molecular weights, according to a previous paper in this series (4). The proteins synthesized in mitochondria after 4 h of cell exposure to the drug show substantially the same electrophoretic distribution, in terms of position and relative proportions of the discrete components, as the products synthesized after a very short period (5 min) of emetine treatment. Profiles of material labeled after periods of cell exposure to the drug longer than 4 h are also qualitatively very similar (see, for example, Fig. 2*d*), although there is a somewhat higher heterogeneous background, which tends to reduce the resolution of the discrete components.

Thus, it appears from the data presented above that HeLa cell mitochondria synthesize the same proteins at approximately the same relative rates, although at declining absolute rates, for a considerable length of time (at least 24 h) after protein synthesis in the cytoplasmic compartment has been essentially completely blocked (12).

Stability of Mitochondrial Protein Products Synthesized after Short Exposure of Cells to Cycloheximide or Emetine—In order to investigate the metabolic stability of the products of mitochondrial protein synthesis under conditions of cell growth and presumably, therefore, of physiological metabolism, HeLa cells were labeled for 15 min with [^{3}H]isoleucine in the presence of cycloheximide, and then chased for various lengths of time in cycloheximide-free medium. Fig. 3*a* shows the profile of the labeled products of mitochondrial protein synthesis immediately after the pulse. The labeling of the discrete components is completely suppressed by chloramphenicol added to the medium 5 min before the pulse, whereas most of the heterogeneous background appears to be chloramphenicol-resistant. In Fig. 3, *Panels b, c,* and *d* show the profiles of the sodium dodecyl sulfate lysates of mitochondrial fractions prepared from cells chased, respectively, for 1, 24, and 72 h.

From the above-described profiles and from others, not shown, pertaining to intermediate chasing times, it was possible to estimate, as illustrated in Fig. 3*a*, the amount of chloramphenicol-sensitive radioactivity associated with each of the major electrophoretic components. The plot of the amount of radioactivity in components 2, 3, 5, and 8 as a function of the length of the chase, expressed as a per cent of the value for

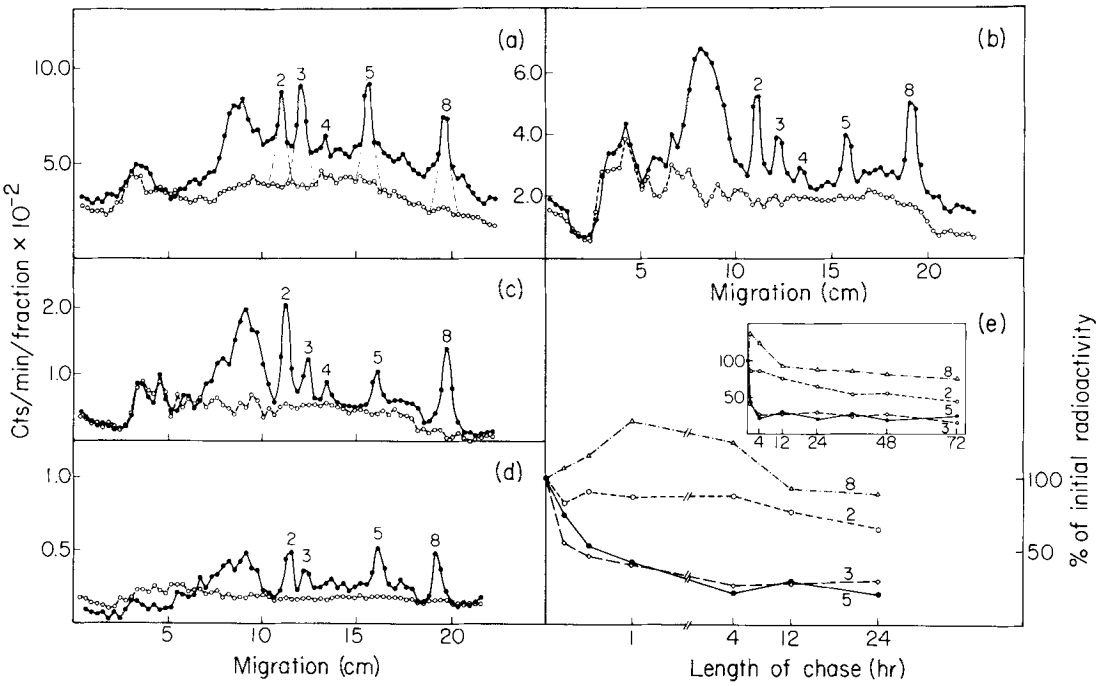

FIG. 3. Electrophoretic distribution of the products of mitochondrial protein synthesis from cells labeled for 15 min with [^{3}H]isoleucine in the presence of cycloheximide and chased for various lengths of time in the absence of the drug. HeLa cells (2.5×10^6 cells/ml) were labeled for 15 min with 12 μCi of [^{3}H]isoleucine/ml (50 Ci/mmol) in isoleucine-free medium (4) in the presence of 100 μg/ml of cycloheximide and in the absence or presence of 100 μg/ml of chloramphenicol, as described elsewhere (4). Cold isoleucine, 10^{-2} M, was then added, the cells were washed at 37° in modified Eagle's medium containing 10^{-2} M cold isoleucine with or without 100 μg/ml of chloramphenicol, resuspended in regular growth medium with or without 40 μg/ml of chloramphenicol, and finally divided into various batches. The chases were interrupted by rapid cooling of the cultures. The mitochondrial fractions were prepared, sonicated, lysed with sodium dodecyl sulfate, and electrophoresed for 40 h. *a*, *b*, *c*, and *d*, profiles of the sodium dodecyl sulfate-lysed mitochondrial fractions prepared immediately after the labeling (*a*) or after chase for 1, 24, and 72 h (*b*, *c*, and *d*, respectively). ●——●, [^{3}H]radioactivity incorporated in the absence of chloramphenicol; ○- - -○, [^{3}H]radioactivity incorporated in the presence of chloramphenicol. *e*, curves illustrating the behavior with time of the radioactivity associated with the individual electrophoretic components. The radioactivity in each peak was estimated by prolonging its contour to a baseline represented by the chloramphenicol-resistant incorporation, as exemplified in *Panel a*. ○- - -○, component 2; ◇- - -◇, component 3; ●——●, component 5; △-·-·-△, component 8. The different samples applied onto the gels contained 120 (*a*), 170 (*b*), 470 (*c*), and 500 (*d*) μg of protein. The counts (*cts*) per min/fraction are normalized in all panels to 1 mg of protein of the mitochondrial fraction.

each peak at the beginning of the chase, is shown in *Panel e* of Fig. 3 and in the *inset*. These values were calculated by taking into account the dilution of specific activity of the mitochondrial membranes due to cell growth during the chase. Up to about 12 h, the metabolic behavior is not the same for all the components analyzed. There is in fact an increase, in the 1st h, in the radioactivity associated with peak 8, followed by a progressive decline to the initial value by the 12th h, while the radioactivity associated with peak 2 shows a slow decrease during the same time; on the other hand, the labeled components 3 and 5 exhibit a rapid and marked decline, so that after 30 min of chase only about 50% and after 4 h only about 25% of the initial radioactivity is still detectable in these components. After these initial differences, all the discrete components are relatively stable for at least three cell generations. It is also interesting to note in the electrophoretic profiles of Fig. 3 the progressive reduction, with increasing lengths of chase, in the absolute level of the chloramphenicol-resistant background.

A pulse-chase experiment similar to that described above was carried out by utilizing emetine, instead of cycloheximide, to block cytoplasmic protein synthesis. Under these conditions, as we have seen above, the block of cytoplasmic protein synthesis continues after removal of the drug and no cell growth occurs throughout the length of the chase. Fig. 4 shows the electrophoretic profiles of the sodium dodecyl sulfate lysates of the mitochondrial fractions prepared immediately after the labeling step (*Panel a*), and after 1 (*Panel b*) or 24 h (*Panel c*) from the beginning of the chase. In Fig. 4, *Panel d*, the relative amounts of radioactivity associated with peaks 2, 3, 5, and 8, estimated as explained in the legend, as well as the total acid-precipitable radioactivity associated with the mitochondrial fraction, are shown as a function of the length of the chase. By comparison with the experiment carried out with cycloheximide, it is quite evident that the metabolic properties of the mitochondrially synthesized proteins are deeply altered after emetine block of cytoplasmic protein synthesis. The most striking phenomenon is the apparent stabilization, in the emetine experiment, of component 5. Components 2 and 8 are, by contrast, apparently destabilized with respect to the cycloheximide experiment. As a result, the electrophoretic profile of the products of mitochondrial protein synthesis is completely altered, with peak 5 becoming progressively more and more dominant with respect to the others (see Fig. 4*c*).

Identical results to those described above were obtained in another experiment in which the chase was carried out in the presence of emetine.

Accumulation of Radioactivity in Mitochondrial Protein Products during Continuous Labeling in Presence of Emetine—The accumulation of radioactivity in the products of mitochondrial protein synthesis was analyzed as a function of the exposure of the cells to [^{3}H]isoleucine in the presence of emetine. As shown in Fig. 5*a* (*solid line*), the specific activity

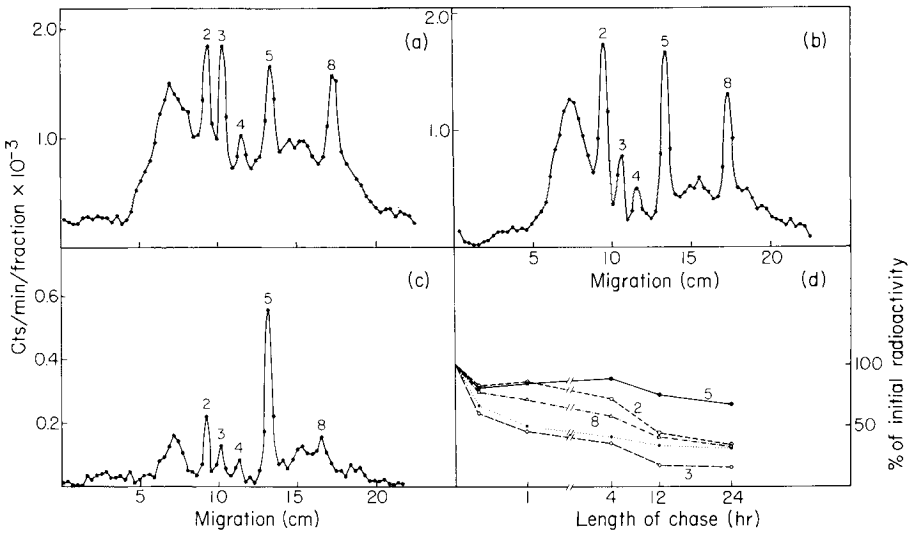

FIG. 4. Electrophoretic distribution, after 40-h runs, of the mitochondrial products from cells labeled for 15 min with [^{3}H]isoleucine in the presence of emetine and chased for various lengths of time in the absence of the drug. The experimental procedure is identical to that described in the legend to Fig. 3, except that emetine (100 μg/ml) was used instead of cycloheximide. *a*, *b*, and *c*, profiles pertaining to the mitochondrial fractions prepared immediately after the labeling (*a*) or after 1 and 24 h of chase (*b* and *c*, respectively). *d*, curves illustrating the behavior with time of the radioactivity associated with the individual components, derived as described in the legend of Fig. 3, except that the base-line utilized was estimated to be equal to the heterogeneous background in the upper portion of the gel. ○- - -○, component 2; ◇- - -◇, component 3; ●——●, component 5; △-·-·-△, component 8; ●· · · ·●, total acid-precipitable radioactivity associated with the mitochondrial fraction. The samples applied onto the gels contained about 200 μg of protein. The counts (*cts*) per min/fraction are normalized in all panels to 1 mg of protein of the mitochondrial fraction.

of the proteins of the mitochondrial fraction does not increase linearly with the time of exposure of the cells to [^{3}H]isoleucine, but reaches an apparent maximum around 4 h and decreases gradually over the next 44 h. (This is not due to exhaustion of the isotope in the medium; of the free [^{3}H]isoleucine present in the medium after 15 min of labeling, more than 95% was recovered in the supernatant after centrifugation of cells labeled for 24 h.) In another experiment, the accumulation of chloramphenicol-sensitive radioactivity was measured, as a function of the time of exposure of the cells to [^{3}H]isoleucine in the presence of emetine, in the total cell lysate. While the initial behavior (during the first 4 to 5 h) of the accumulation curve thus obtained was very similar to that observed for the isolated mitochondrial fraction (Fig. 5*a*), an apparent plateau was observed for longer labeling times (*dashed line*). A progressively decreasing contribution to the total labeling by the nonmitochondrial membrane components of the mitochondrial fraction (6), or a preferential disintegration of altered mitochondria could account for the discrepancy between the two curves. It is significant, however, that both in total cells and in the crude mitochondrial fraction, no net increase in the specific activity of the products of mitochondrial protein synthesis as a whole was observed after 4 to 5 h of exposure of the cells to [^{3}H]isoleucine in the presence of emetine. This phenomenon will be analyzed in more detail below.

Fig. 5, *b*, *c*, and *d*, shows the electrophoretic profiles of the products of mitochondrial protein synthesis from cells labeled for 1, 4, and 24 h, respectively. These profiles are quite similar up to at least 4 h of continuous labeling; after 24 h, an increase in the proportion of radioactivity associated with component 5 is observed (Fig. 5*d*). In agreement with the shape of the curve of accumulation of radioactivity shown in Fig. 5*a* (*solid line*), there is no increase in the specific activity of any of the components after 4 h of exposure of the cells to [^{3}H]isoleucine in the presence of emetine (Fig. 5*e*).

As shown above, the rate of mitochondrial protein synthesis remains at a level greater than 25% of the maximum for a long period of time (at least 20 h) after the first 4 h of emetine treatment; by contrast, the accumulated radioactivity ceases to increase fairly suddenly after about 4 h of drug treatment. This observation suggested a rapid increase, around that time, in the rate of decay of newly synthesized molecules. This possibility has been directly verified by the experiment shown in Fig. 6. HeLa cells, exposed to emetine for 4 h, were labeled with [^{3}H]isoleucine for 30 min in the presence of the drug, and then incubated in fresh medium containing emetine for 20 h. None of the components which were labeled 4 h after addition of emetine (Fig. 6*a*) is clearly recognizable in the electrophoretic pattern obtained after 20 h of chase in the presence of the drug (Fig. 6*b*). Thus, it seems that the proteins synthesized in mitochondria late (after the first 4 h) during the exposure of the cells to emetine are all significantly less stable than the ones synthesized early (after 5 min, see Fig. 4), despite the similarity of their electrophoretic distributions.

Metabolism of Mitochondrial Protein Products in HeLa Cells Previously Grown for Prolonged Time in Presence of Chloramphenicol—It has been previously shown in this laboratory that HeLa cells can grow at a normal rate for at least two generations in the presence of concentrations of chloramphenicol capable of inhibiting, essentially completely, mitochondrial protein synthesis (20). Upon removal of the drug, protein synthesis appeared to be restored fairly rapidly (within 30 min) in the mitochondrial compartment (20). In order to investigate the effects of a prolonged block of mitochondrial protein synthesis on the metabolism of the proteins synthesized in the organelles after the removal of chloramphenicol, the experiments described in the previous sections were repeated on HeLa cells which had been grown for 40 h in the presence of the drug. In all cases, the cells were washed free of chloramphenicol, equilibrated for 30 min with drug-free medium, and then subjected to the particular experimental conditions to be investigated.

In yeast (21) and *Neurospora crassa* (22), the rate of mitochondrial protein synthesis has been reported to be greatly

stimulated by prior incubation of the cells in chloramphenicol. By contrast, in this work, as in the earlier work on HeLa cells (20), after chloramphenicol pretreatment of the cells, the rate of amino acid incorporation was not reproducibly higher than that observed in control cells. Furthermore, in chloramphenicol-pretreated cells, exposure to emetine caused a decline in the rate of mitochondrial protein synthesis very similar to that found in the case of normally grown cells (Fig. 7*a*). The electrophoretic distribution of the mitochondrial protein products labeled with [^{3}H]isoleucine for 30 min after different times of emetine treatment in chloramphenicol-treated cells shows the same general characteristics observed in material from control cells; however, there appear to be differences in the relative abundance of the various labeled components; in particular, the relative labeling of component 5 is markedly increased (Fig. 7, *b*, *c*, and *d*).

The effects of chloramphenicol pretreatment of the cells on the stability of the mitochondrial products labeled for 15 min with [^{3}H]isoleucine in the presence of cycloheximide and then chased in drug-free medium are evident by comparison of the diagrams in Figs. 3 and 8. Apart from the newly synthesized component 8, which appears to be destabilized, all other major components are more stable after chloramphenicol pretreatment. This effect is especially striking in the case of component 5, whose radioactivity does not show the initial rapid decline observed in the case of normally grown cells, but remains constant for at least 24 h. A pulse-chase experiment utilizing emetine instead of cycloheximide to block cytoplasmic protein synthesis was also performed on chloramphenicol pretreated cells. The electrophoretic distribution of the mitochondrial products labeled for 15 min with [^{3}H]isoleucine in the presence of emetine evolves during the chase (Fig. 9) in substantially the same way as in the case of normally grown cells (Fig. 4). The curves representing the quantitative behavior of the individual electrophoretic components (Fig. 9*d*) do not differ substantially from the ones shown in Fig. 4*d*.

The overall curve of accumulation of radioactivity in the mitochondrial fraction of chloramphenicol-pretreated cells, as a function of the exposure of the cells to [^{3}H]isoleucine in the presence of emetine (Fig. 10*a*, *filled circles, solid line*), does not show any substantial difference from that obtained for normally grown cells (Fig. 5*a*). By contrast, as illustrated in Fig. 10, *b* to *e*, striking alterations take place, with increasing labeling times, in the relative amounts of radioactivity associated with the various mitochondrial products. Already after 1 h of labeling, one can observe a disproportion between the radioactivity associated with component 5 and that present in the other mitochondrial products (compare, for example, Figs. 5*b* and 10*b*); component 5, after 24 h of labeling, becomes the only recognizable peak in the electrophoretic pattern of the mitochondrial fraction (Fig. 10*d*). This accumulation of radioactivity in component 5 could also be observed if cycloheximide was used, instead of emetine, to block cytoplasmic protein synthesis (Fig. 10*f*).

The stability of the mitochondrial protein products synthesized after 4 h of exposure to emetine was also investigated in chloramphenicol-pretreated cells. As shown in Fig. 6, *c* and *d*, unlike the case of control cells, a substantial fraction (about 40%) of the radioactivity incorporated into component 5 during a 30-min pulse given 4 h after block of cytoplasmic protein synthesis is still present in this peak after 20 h of chase in the presence of emetine; this indicates that, in chloramphenicol-pretreated cells, in contrast to normal cells, the stabilization of component 5 involves material synthesized over a period of time longer than 4 h. The fate of the other mitochondrial components is, on the contrary, analogous under the two experimental conditions.

That, also in the case of chloramphenicol-treated cells, most of the radioactivity measured after long exposure of the cells to [^{3}H]isoleucine is due to mitochondrial products synthesized during the first few hours of labeling, was shown by another experiment, described below. HeLa cells, grown for 40 h in the presence of chloramphenicol, were labeled with [^{3}H]isoleucine for 4 h in the presence of emetine. The culture was then divided into two portions: one-half of the labeled cells was left in the same medium; the other half was transferred into label-free medium containing emetine. In this way, the first portion was labeled continuously for 48 h, while the second one was labeled for the first 4 h and chased in the presence of emetine for the subsequent 44 h. The chloramphenicol-sensitive portion of the incorporated radioactivity, measured in whole cell lysates at various intervals before and after the splitting of the cultures, is plotted in Fig. 10*a*. The chase curve generated at 4 h (Fig. 10*a*, *filled triangles, solid line*) is virtually indistinguishable from the curve of continuous labeling (Fig. 10*a*, *open circles, dashed line*). This result supports the idea that, in the period between 4 and 48 h, no appreciable contribution to the total acid-precipitable radioactivity comes from mitochondrial products synthesized after 4 h of exposure of the cells to emetine, although mitochondrial protein synthesis does continue for at least 24 h after the addition of the drug (Figs. 2 *a* and 7*a*). The mitochondrial fractions prepared from the two cultures used in this experiment were electrophoresed, and the two patterns obtained are shown, superimposed, in Fig. 10*g*; they are qualitatively and quantitatively identical. Thus, most of the radioactivity associated with peak 5 after 48 h of labeling is due to synthesis which occurred in the first few hours after addition of emetine. This implies that the residual radioactivity in component 5 observed after 20 h of chase (Fig. 6*d*) must decay to a negligible level over the next 24 h.

All the experiments described in this section were carried out with cells preincubated with chloramphenicol for 40 h. However, the effect of chloramphenicol on mitochondrial protein metabolism could be observed after much shorter times of pretreatment of the cells with this drug. From Fig. 11 it appears that even a 15-min cell exposure to chloramphenicol affects to some extent the stability of the mitochondrial products. After 4 to 12 h of chloramphenicol treatment, the newly synthesized component 5 shows a stability comparable to that seen after 40 h of treatment (compare Fig. 10*c* with Fig. 11*c*).

DISCUSSION

In the present work, we have investigated the metabolic behavior of the products of mitochondrial protein synthesis in HeLa cells, focusing our attention on the four major individual electrophoretic components, *i.e.* components 2, 3, 5, and 8, previously identified among such products; these have an estimated molecular weight of 39,000, 35,000, 27,500, and 19,500, respectively (4). Although it is not yet known whether or not more than one species is present in some of these electrophoretic components, the metabolic properties studied here probably reflect those of the major polypeptide constituent(s) of each peak.

In vivo mitochondrial protein synthesis can only be studied by blocking cytoplasmic protein synthesis, and this block has been found here to have a considerable effect on the rate of mitochondrial protein synthesis and on the stability of its products. Therefore, we have made an effort to discriminate

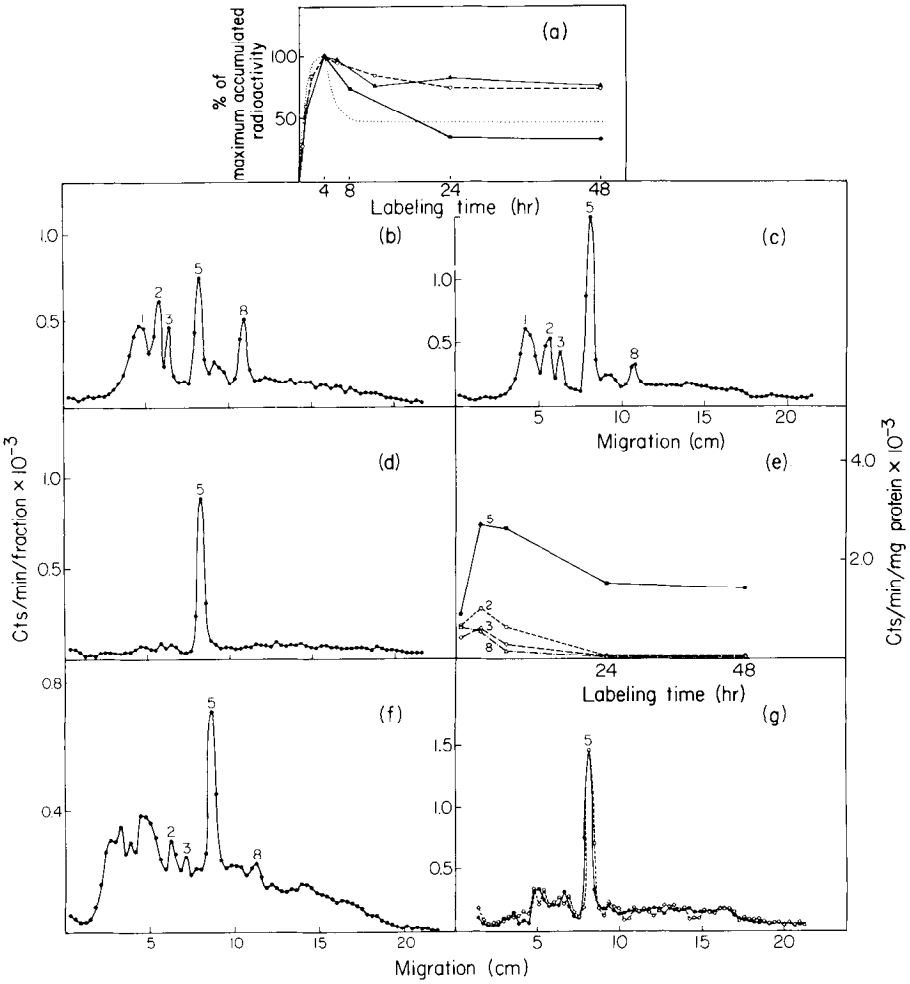

FIG. 10. *a*, kinetics of accumulation of radioactivity in the products of mitochondrial protein synthesis after prolonged growth of the cells in the presence of chloramphenicol. Experimental conditions as in Fig. 5*a*, except that the cells were grown for 40 h in the presence of chloramphenicol (40 μg/ml), washed, and equilibrated with drug-free medium for 30 min prior to the labeling. ●——●, total acid-precipitable radioactivity associated with the mitochondrial fraction (100% ≃ 26,000 cpm (*cts/min*)/mg of protein); ○- - -○, chloramphenicol-sensitive acid-precipitable radioactivity associated with the whole cell lysate; ▲——▲, chloramphenicol-sensitive acid-precipitable radioactivity associated with the whole cell lysate after a 4-h labeling and subsequent chase (see text); · · · ·, theoretical curve calculated as described in the text and under "Appendix" in the miniprint supplement. In all experiments described above, the radioactivity data were normalized for differences in the amount of protein in different samples. *b* to *e*, electrophoretic distribution of the sodium dodecyl sulfate lysates of the mitochondrial fractions from cells grown for 40 h in the presence of chloramphenicol, and then labeled with [^{3}H]isoleucine for various lengths of time in the presence of 100 μg/ml of emetine. Portions of the lysates of the mitochondrial fractions corresponding to some of the time points of the experiment described in *Panel a* (●——●) were electrophoresed for 24 h. *b*, *c*, and *d*, profiles of the fractions prepared after 1, 4, and 24 h of labeling, respectively. *e*, curves illustrating the behavior with time of the radioactivity associated with the individual components, derived as described in the legend to Fig. 4. ○- - -○, component 2; ◇- - -◇, component 3; ●——●, component 5; △-·-·-△, component 8. *f*, electrophoretic profile (24-h run) of the sodium dodecyl sulfate lysate of the mitochondrial fraction from cells grown for 40 h in the presence of chloramphenicol and then labeled with [^{3}H]isoleucine for 24 h in the presence of 100 μg/ml of cycloheximide. *g*, electrophoretic profiles (24-h runs) of the sodium dodecyl sulfate lysates of the mitochondrial fractions from cells which had been grown for 40 h in the presence of chloramphenicol, and then either labeled with [^{3}H]isoleucine for 48 h in the presence of emetine (○- - -○), or labeled for 4 h and chased for 44 hours in the presence of the same drug (●- - -●). The different samples applied onto the gels contained between 250 and 500 μg of protein. The counts per min/fraction are normalized in all panels to 1 mg of protein of the mitochondrial fraction.

between the physiological phenomena and the effects of inhibition of cytoplasmic protein synthesis. Furthermore, we have investigated the influence of a prolonged block of mitochondrial protein synthesis on the metabolic behavior of its products synthesized after removal of the drug.

Effect of Emetine and Cycloheximide on Mitochondrial Protein Synthesis—Exposure of HeLa cells to 100 μg/ml of emetine results in a rapid decrease in the rate of labeling of the mitochondrial translation products with radioactive isoleucine, affecting in a parallel way all the discrete electrophoretic components. This decline does not seem to be due to a direct effect of the drug on mitochondrial protein synthesis, an effect which has been reported in other systems (17–19), since a similar rapid decline was observed also in the presence of 100 μg/ml of cycloheximide, an inhibitor of cytoplasmic protein synthesis which has apparently little or no direct effect on organelle-specific protein synthesis (17–19).

In HeLa cells, cycloheximide inhibition of cytoplasmic protein synthesis causes a fairly rapid decrease in the rate of mitochondrial RNA synthesis, with a half-life of about 2 h (23). The effect of a block of cytoplasmic protein synthesis on the rate of mitochondrial protein synthesis could thus be due to a

depletion of the pool of mitochondrial DNA-coded, poly(A)-containing RNA species (24), which are presumably mRNA species, if the latter were very unstable. However, this explanation seems unlikely, since enucleation experiments strongly suggest that the mRNA species translated in mitochondria of a monkey cell line have a considerable stability.[1]

A more plausible interpretation is that the effect on mitochondrial protein synthesis is due to cessation of synthesis, and consequent progressive exhaustion of the existing pools, of cytoplasmic protein(s) essential for the synthesis and integration into the inner membrane of the mitochondrially synthesized polypeptides (3). In the absence of such cytoplasmic protein(s), mitochondrial protein synthesis would proceed at a much lower rate and would yield products which are not integrated into the inner membrane.

If the above interpretation is correct, it would appear that in exponentially growing HeLa cells there is a pool of these essential cytoplasmic protein(s) which is sufficient to support the synthesis of the mitochondrial products at a normal rate for less than 1 h. These cytoplasmic protein(s) may perform some basic function required for the synthesis of all the mitochondrially made polypeptides of the inner membrane, acting directly at the level of their polymerization or at the level of their integration into the inner membrane, or both, if integration is somehow coupled with synthesis (3). The reason why mitochondrial protein synthesis decays almost completely after 12 h of cycloheximide treatment, while a residual 30% of synthesis is observed for at least 24 h of cell exposure to emetine, is as yet unclear.

Stability of Products of Mitochondrial Protein Synthesis – Since the effects of cycloheximide on cytoplasmic protein synthesis (8, 15) and on cell growth (present work) are immediately reversible upon removal of the drug, it was possible to study here the stability of the labeled products of mitochondrial protein synthesis under close to physiological conditions, by pulse labeling HeLa cells with an amino acid in the presence of cycloheximide and then chasing them in drug-free medium.

Considerable differences were observed, during the first 12 h of chase, in the metabolic stability of the main electrophoretic components. Especially conspicuous was the decay of the radioactivity associated with components 3 and 5. After the first 12 h of chase, all discrete electrophoretic components showed a very slow decline in radioactivity during the next 3 days.

A plausible, though not exclusive, interpretation of these results is that the relatively stable portion of the products of mitochondrial protein synthesis represents those molecules which are incorporated into the inner mitochondrial membrane, and that integration into the membrane requires combination of the mitochondrially made subunits of the various enzymatic complexes with the corresponding cytoplasmically made subunits. If this is so, one must conclude that a major fraction of the newly synthesized components 3 and 5 is not incorporated into functional complexes of the inner membrane and is therefore degraded.

A profound change in the stability of the discrete products of mitochondrial protein synthesis occurs in the continued absence of cytoplasmic protein synthesis, as is the situation when emetine is used as an inhibitor during the pulse labeling. Of the four main electrophoretic components analyzed, components 2 and 8 appear to be considerably less stable under these conditions than in the presence of cytoplasmic protein synthesis, suggesting that continued renewal of some cytoplasmic proteins is required for their stability. Most striking, however, is the apparent stabilization, relative to the physiological situation, of component 5; this phenomenon will be discussed below.

The products of mitochondrial protein synthesis pulse labeled after 4 h of emetine treatment were found to be considerably less stable than those labeled at the beginning of the drug treatment. It is possible that this is due to their not being integrated into the inner mitochondrial membrane, because of its lack of certain essential cytoplasmically synthesized polypeptide(s). As a result of the instability of the mitochondrial products synthesized after the first 4 h of emetine treatment, their contribution to the curve of accumulation of radioactivity was expected to be negligible. This expectation has indeed been verified experimentally in the case of cells pretreated with chloramphenicol. Furthermore, as shown under "Appendix," in the miniprint supplement, a theoretical curve of accumulation of radioactivity constructed by assuming an infinitely high rate of decay of the molecules newly synthesized after the first 4 h of emetine treatment was found to reproduce fairly closely the characteristics of the experimental one.

Effects of a Block of Mitochondrial Protein Synthesis on Subsequent Metabolism of Mitochondrial Protein Products – It has been previously observed that mitochondrial protein synthesis occurs at a normal or near to normal rate in HeLa cells pretreated for up to 7 days (about 4.5 cell generations) with 40 μg/ml of chloramphenicol (20). This implies a continuing formation of a functional mitochondrial protein-synthesizing apparatus, and a continuous growth and division of mitochondria and gross assembly of the inner mitochondrial membrane (20, 25).

The lack of any stimulating effect of chloramphenicol treatment of the cells, even prolonged, on the subsequent rate of organelle-specific protein synthesis has been confirmed in the present work. On the contrary, exposure of the cells to chloramphenicol for even a relatively short time has been found here to affect significantly the physiological stability of the mitochondrial products pulse-labeled after chloramphenicol removal. Apart from component 8, which was less stable under these conditions, all other major components appeared to be stabilized. This effect was especially marked in the case of component 5, which was stable for at least 24 h, instead of showing the sharp initial decline observed in normally grown cells (see below).

Special Metabolic Behavior of Component 5 – The metabolic behavior of the electrophoretic component 5, or of a major polypeptide constituent of it, differs markedly from that of all other components. The observed degradation, under conditions of cytoplasmic protein synthesis, of a large fraction of component 5 labeled in a 15-min pulse in the presence of cycloheximide and chased in the absence of the drug does not, probably, result from the exhaustion, during the pulse, of a very small pool of cytoplasmic protein(s) necessary for its integration; we have shown, in fact, that the newly synthesized component 5 is completely stable if emetine is used instead of cycloheximide. Rather, the data would be consistent with the idea that some step other than the synthesis of these essential cytoplasmic proteins (transport through the membrane?) is rate-limiting in the stabilization of component 5. Evidence for the transport of cytoplasmically synthesized proteins into mitochondria being a step distinct from their synthesis has recently been reported for *Neurospora crassa* (26). Whatever the rate-limiting step involved is, component 5 ap-

[1] J. M. England, P. Costantino, and B. Attardi, manuscript in preparation.

pears to be normally synthesized in excess of its utilization. This excess of synthesis would provide a mechanism of adaptation, whereby a varying proportion of the synthesized component may be integrated into the membrane and, presumably therefore, stabilized, depending upon the physiological needs. It is suggested here that the size of the pool of the putative essential cytoplasmic component(s) plays a role in this regulation. Under conditions of inhibition of mitochondrial protein synthesis, the size of this pool would become larger, thus allowing the subsequent utilization of a larger than normal fraction of the newly synthesized component 5 upon block of cytoplasmic protein synthesis. The dependence of the chloramphenicol effect on the length of the pretreatment of the cells with this drug is in agreement with the idea of a progressive enlargement of this hypothetical pool.

The results of the pulse-chase experiment carried out with emetine on control cells suggest that complete and irreversible inhibition of cytoplasmic protein synthesis may lead, by feedback effect, to the stabilization of the newly synthesized component 5. The observed difference in behavior of component 5 in the comparable pulse-chase experiment carried out with cycloheximide may be due to the reversibility of the block of cytoplasmic protein synthesis by the latter drug. In agreement with this idea is the observation that a stabilization of component 5 also occurred in experiments of continuous labeling in the presence of cycloheximide, in cells pretreated with chloramphenicol.

It seems likely that the distinctive metabolic behavior of component 5 reflects a physiological situation and does not result from abnormalities produced by the use of inhibitors; as a matter of fact, this effect was already apparent at a time when the block to cytoplasmic protein synthesis had not yet caused, presumably, any irreversible damage to the cells, as judged from the reversibility of the cycloheximide effects.

CONCLUSIONS

Previous work on mitochondrial protein synthesis in yeast has emphasized the role of cytoplasmically made proteins in controlling the rate of mitochondrial protein synthesis (see review by Schatz and Mason (3)). This role has been confirmed in the present work with HeLa cells by the progressive inhibition of organelle-specific protein synthesis during continuous exposure of the cells to emetine or cycloheximide.

The most striking effects detected in the present work, and not previously reported by others, concern the role of cytoplasmically made proteins in stabilizing the mitochondrial products. It seems possible that this stabilizing role pertains specifically to the cytoplasmically made subunits of the various enzymatic complexes of the inner membrane, which would combine with the corresponding mitochondrially made subunits and, thus, allow their proper integration into the membrane.

The various mitochondrial protein products analyzed here have revealed marked differences in their metabolic behavior, as evidenced by their different stability under physiological conditions and by the differential effects on their stability of an inhibition of either cytoplasmic or mitochondrial protein synthesis. This suggests that the synthesis and stability of each of these components is subject to a different regulation. In *Neurospora crassa*, the difference in the pool size of the three mitochondrially synthesized subunits of the cytochrome oxidase (27) suggests a possible differential modulation of the metabolism of the three polypeptides.

The picture of mitochondrial protein synthesis which starts emerging from this work and previous work on *Neurospora crassa* and yeast is one in which the synthesis and stability of the individual components is finely and differentially modulated in response to physiological needs, with a complex regulatory interplay between mitochondrial and cytoplasmic protein synthesis.

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Miniprint to
"Metabolic Properties of the Products of Mitochondrial Protein Synthesis in HeLa Cells" by Paolo Costantino and Giuseppe Attardi

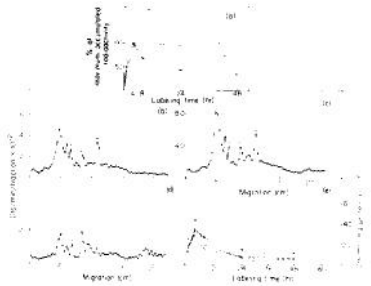

FIG. 5. (a) Kinetics of accumulation of radioactivity in the products of mitochondrial protein synthesis in control cells. HeLa cells (1.5×10^6/ml) were labeled with 5 μCi/ml [^{3}H]isoleucine in isoleucine-free medium (4) in the presence of emetine (100 μg/ml) and in the presence or absence of chloramphenicol (100 μg/ml) for various lengths of time. The total acid-precipitable radioactivity associated with the mitochondrial fraction (●—●) (100% = 20,000 cts/min/mg protein), or the chloramphenicol-sensitive acid-precipitable radioactivity associated with the whole cell lysate (o—o) (100% = 4000 cts/min/mg protein; chloramphenicol-sensitivity = 55%) is plotted; ·····, ·····, theoretical curves, calculated as described in the Appendix. (b-e) Electrophoretic distribution of the products of mitochondrial protein synthesis labeled with [^{3}H]isoleucine for various lengths of time in the presence of emetine. Portions of the lysates of the mitochondrial fractions corresponding to some of the time points of the experiment described in panel a (●—●) were electrophoresed for 24 hours. b,c,d: Profiles of the fractions prepared after labeling for 1, 4 and 24 hours, respectively. e: Curves illustrating the behavior with time of the radioactivity associated with the individual components, derived as described in the legend to Fig. 4. ◇—◇, component 5; ♦—♦, component 5; o···o, component 8. The different samples applied onto the gels contained between 480 and 600 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction.

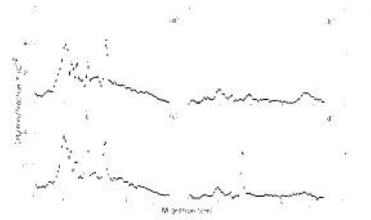

FIG. 6. Profiles of 24 hour-long electrophoretic runs of the mitochondrial products labeled for 30 min with [^{3}H]isoleucine 4 hours after the addition of emetine, or labeled and then chased for 20 hours in the presence of the drug, in cells which had been grown in the absence (a, b) or for 40 hours in the presence of chloramphenicol (c, d). a,b: HeLa cells were exposed to 100 μg/ml of emetine for 4 hours, then [^{3}H]isoleucine (>10 μCi/ml) was added and labeling carried out for 30 min in isoleucine-free medium (4). One-half of the cells was harvested, while the other half was chased, in the presence of emetine, for 20 hours, as described in the legend to Fig. 3. a: Mitochondrial fraction prepared immediately after the labeling. b: Mitochondrial fraction prepared after 20 hours of chase. c,d: Experimental conditions as above, except that the cells used had been grown for 40 hours in the presence of 40 μg/ml of chloramphenicol. c: Mitochondrial fraction prepared immediately after the labeling. d: Mitochondrial fraction prepared after 20 hours of chase. The different samples applied onto the gels contained between 300 and 550 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction.

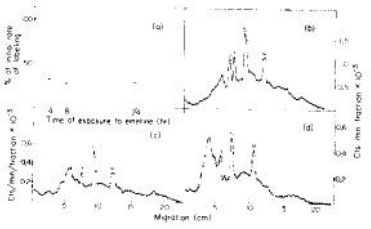

FIG. 7. (a) Kinetics of decline of the rate of mitochondrial protein synthesis in the presence of emetine after prolonged treatment of the cells with chloramphenicol. Experimental conditions as described in Fig. 2a, except that the cells were grown for 40 hours in the presence of 40 μg/ml chloramphenicol, washed and equilibrated with drug-free medium for 30 min prior to the addition of emetine. ●—●, total acid-precipitable radioactivity associated with the mitochondrial fraction (100% = 7800 cts/min/mg protein). (b,c,d) Electrophoretic profiles of the products of mitochondrial protein synthesis pulse labeled with [^{3}H]isoleucine after 5 min, 4 hours or 12 hours of exposure to emetine, in cells which had been grown for 40 hours in the presence of chloramphenicol. 24 hour-long electrophoretic runs of the sodium dodecyl sulfate-lysed mitochondrial fractions from cells labeled with [^{3}H]isoleucine for 30 min after 5 min (b) or 4 hours (c) of emetine treatment (corresponding to two of the time points of the experiment described in panel a), or for 1 hour after 12 hours exposure to the drug (d). The samples applied onto the gels contained about 600 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction.

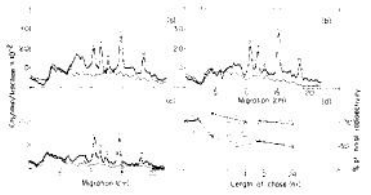

FIG. 8. Electrophoretic distribution, after 40-hour runs, of the sodium dodecyl sulfate lysates of the mitochondrial fractions from cells grown for 40 hours in the presence of chloramphenicol, then labeled for 15 min in the presence of cycloheximide and chased for various lengths of time in the absence of the drug. The experimental procedure is identical to that described for Fig. 5, except that cells grown for 40 hours in the presence of 40 μg/ml of chloramphenicol were utilized. a,b,c: Mitochondrial fractions prepared immediately after the labeling (a), or after 1 (b) or 24 hours (c) of chase. d: Curves describing the behavior with time of the radioactivity associated with the individual components, derived as described in the legend to Fig. 5. o···o, component 2; ◇···◇, component 5; ♦—♦, component 5; Δ·····Δ, component 8. The different samples applied onto the gels contained between 480 and 580 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction.

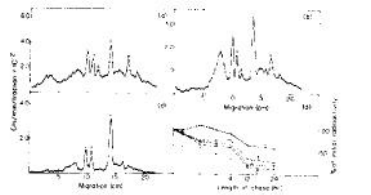

FIG. 9. Electrophoretic distribution, after 40-hour runs, of the sodium dodecyl sulfate lysates of the mitochondrial fractions from cells grown for 40 hours in the presence of chloramphenicol, then labeled for 15 min in the presence of emetine and chased for various lengths of time in the presence of this drug. The experimental procedure is identical to that described in the legend to Fig. 4, except that cells grown for 40 hours in the presence of 40 μg/ml of chloramphenicol were utilized. a,b,c: Mitochondrial fractions prepared immediately after the labeling (a) or after 1 (b) or 24 hours (c) of chase. d: Curves describing the behavior with time of the radioactivity associated with the individual components, derived as described in the legend to Fig. 4. o···o, component 2; ◇···◇, component 5; ♦—♦, component 5; Δ·····Δ, component 8. The different samples applied onto the gels contained between 280 and 550 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction.

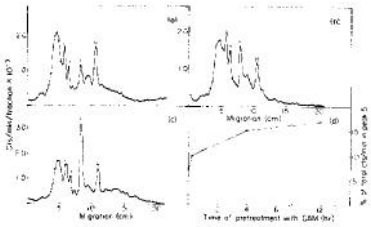

FIG. 11. Electrophoretic distribution, after 24-hour runs, of the sodium dodecyl sulfate lysates of the mitochondrial fractions from cells grown for various lengths of time in the presence of chloramphenicol, and then labeled for 4 hours with [^{3}H]isoleucine in the presence of emetine in isoleucine-free medium (4). HeLa cells were grown for various lengths of time in the presence of 40 μg/ml of chloramphenicol, the cells were then washed, concentrated to 1.5×10^6 cells/ml and equilibrated with drug-free medium for 30 min. Emetine was then added and labeling carried out for 4 hours with 5 μCi/ml [^{3}H]isoleucine. a,b,c: Mitochondrial fractions from control cells (a) and from cells grown for 15 min (b) or 12 hours (c) in the presence of chloramphenicol. d: Curve representing the fraction of the total radioactivity in the electrophoretic patterns which is associated with component 5, as a function of the time of pre-exposure of the cells to chloramphenicol. The data were derived from the profiles shown and from others pertaining to intermediate times of exposure of the cells to chloramphenicol. The different samples applied onto the gels contained between 300 and 350 μg protein. The cts/min/fraction are normalized in all panels to 1 mg protein of the mitochondrial fraction. CAM = chloramphenicol.

APPENDIX

Theoretical Determination of the Kinetics of Accumulation of Radioactivity in the Mitochondrial Protein Products in the Presence of Emetine

Massimo Testa, Paolo Costantino and Giuseppe Attardi

In cells which are exposed to a labeled amino acid present in non-limiting amounts, the accumulation of radioactivity in a given protein over the period 0-T is a function of both the instantaneous rate of incorporation [i(t)] of the isotope over the same period of time and of the decay of the molecules synthesized at the time t over the period t-T.

In the present case, the total labeling of the mitochondrial fraction during a 30 min pulse with [^{3}H]isoleucine, or the chloramphenicol-sensitive labeling of whole cell lysates during a 15 min pulse with the tritiated amino acid, has been taken as an indicator of the instantaneous rate of incorporation of the isotope at various times during exposure to emetine of normally grown or chloramphenicol pre-treated cells. The experimental data of Figures 2a and 7a were found to be roughly described by

$$i(t) = Ae^{-\frac{t}{\tau_1}} + (1-A), \qquad (1)$$

where A and τ_1 are two appropriately chosen constants.

As concerns the rate of decay of the molecules synthesized at various times during emetine treatment, the experimental data suggested a dramatic decrease in the stability of the newly synthesized molecules after about 4 hours of drug treatment. For the purpose of the present calculations, it was assumed that the kinetics of decay remains approximately the same for molecules labeled during the first 4 hours of emetine treatment. In particular, the shape of the experimental decay curves of Figures 4d and 9d has been approximated by

$$f(T-t) = Ce^{-\frac{(T-t)}{\tau_2}} + (1-C), \qquad (2)$$

where f(T-t) represents the fraction of radioactivity remaining after a time (T-t) from the pulse (with t varying between 0 and T), and C and τ_2 are two appropriate constants. For cell exposures to emetine longer than 4 hours, it has been assumed that the rate of decay of newly synthesized molecules becomes infinitely high, that is f(T-t) = 0.

By the definition of the rate of incorporation i(t), the amount of radioactivity incorporated into mitochondrially synthesized proteins in the infinitesimal time interval between t and t + dt is proportional to i(t)dt. In view of the decay, at the time of observation T, the amount of radioactivity incorporated in this infinitesimal interval will become i(t)dt · f(T-t). The curve of accumulation of radioactivity will then be the sum of all these contributions. If t is < h (time at which the rate of decay becomes infinite, we will have:

$$T \geq h \qquad q(T) = \int_0^T i(t)\, f(T-t)dt \qquad (3)$$

For T > h, the only contributions to q(T) will derive from incorporation occurring at times t > h. Therefore:

$$T \geq h \qquad q(T) = \int_{T-h}^{T} i(t)\, f(T-t)dt \qquad (4)$$

By substituting in (3) and (4) the functions (1) and (2), one obtains:

$$\text{for } T \leq h \qquad q(T) = \left[\frac{AC}{\frac{1}{\tau_2} - \frac{1}{\tau_1}} - A(1-C)\tau_1\right] e^{-\frac{T}{\tau_1}} - \left[\frac{AC}{\frac{1}{\tau_2} - \frac{1}{\tau_1}} + (1-A)C\tau_2\right] e^{-\frac{T}{\tau_2}} + (1-A)T + A(1-C)\tau_1 + (1-A)C\tau_2 \qquad (5)$$

$$\text{for } T \geq h \qquad q(T) = \left\{\frac{AC}{\frac{1}{\tau_2} - \frac{1}{\tau_1}}\left(e^{\left(\frac{1}{\tau_2} - \frac{1}{\tau_1}\right)h} - 1\right)\right\} e^{-\frac{T}{\tau_1}} + (1-A)C\tau_2\left(e^{\frac{h}{\tau_2}} - 1\right) e^{-\frac{T}{\tau_2}} - A(1-C)\tau_1\left(e^{-\frac{h}{\tau_1}} - 1\right) + (1-A)(1-C)h \qquad (6)$$

It can be easily seen that the function defined by equations (5) and (6) has a maximum at T = h. In order to compare the theoretical curve to the experimental data, the theoretical values have been expressed as percentages of q(h). As shown in Figures 5a and 10a, the theoretical curves thus constructed (·····) have the same basic characteristics as the experimental curves of accumulation of radioactivity, thus confirming the validity of the assumptions made here. In particular, one can see that if the assumption of an infinite rate of decay for molecules labeled after time h were not included in the calculation, equation (5), which is a monotonously growing function, would describe the accumulation curve over the entire period of observation (·····) line in Fig. 5a), in clear contrast to the experimental data.