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Photoincorporation of Puromycin into Rat Liver Ribosomes and Subunits†

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ABSTRACT: [^{3}H]Puromycin was covalently incorporated into rat liver ribosomes and isolated 40S and 60S subunits on irradiation at 254 nm. A study of the concentration dependence of this photolytic incorporation suggested that it arose from specific sites on isolated subunits but also from unspecific ones in the case of ribosomes, these sites being probably located on contaminant nonribosomal proteins. Puromycin was incorporated simultaneously into ribosomal proteins and rRNAs. The results from simultaneous one-dimensional and two-dimensional gel electrophoreses showed a small distribution of label among ribosomal proteins in 60S subunits and in 80S ribosomes, L10 being the most radioactive protein. Some antibiotics, which act on the peptidyltransferase center (amicetin and gougerotin), and also tetracycline competed with this labeling. Therefore, it was concluded that puromycin interaction with protein L10 occurred most likely at a functional site. In the case of free 40S subunits, labeling distribution among proteins was much wider. The possibility that proteins S3 and perhaps S23–24, which were significantly labeled in crude ribosomes too, also belong to a specific site interacting with puromycin is discussed.

Coopermann et al. (1975), Coopermann (1980), and Jaynes et al. (1978) reported a photochemical reaction of labeled puromycin with ribosomes and isolated subunits of *Escherichia coli* and especially with rRNAs and proteins located on both subunits. Labeling of these components was observed in puromycin-specific binding sites, in close vicinity to the peptidyltransferase center.

A similar study is carried out here, on the localization of puromycin-incorporation sites within rat liver ribosomes and subunits, and on the concentration dependence of this incorporation. Until now, very few affinity labeling studies have been carried out to locate components involved in the peptidyltransferase center of these ribosomes (Stahl et al., 1974, 1979; Czernilofsky et al., 1977; Böhm et al., 1979). The main advantage of the direct use of puromycin over these other experiments is that it excludes the introduction of an extraneous chemical moiety.

Materials and Methods

Puromycin dihydrochloride was obtained from Sigma. [8-^{3}H]Puromycin (5–5.7 Ci/mmol) and [^{14}C]phenylalanine (200 mCi/mmol) were purchased from Amersham.

Preparation of Ribosomes. Rat liver ribosomes consisting mainly of polysomes were isolated according to a method adapted from that of Moldave & Skogerson (1967). No attempt was made to remove nascent chains and numerous other factors, e.g., aminoacyl-tRNA and messenger RNA, because the washing of ribosomes by high concentrations of salt has a particularly dramatic effect on protein reactivity (Ghosh & Moore, 1979). Occasionally they are denoted as crude ribosomes to distinguish them from the washed ribosomes obtained after puromycin and high-salt treatment used by others in comparable studies (see Discussion). Isolated subunits used directly in photolabeling experiments or subunits prepared from ribosomes labeled with [^{3}H]puromycin were obtained as described by Madjar et al. (1977) using a method adapted from that of Blobel & Sabatini (1971).

Photolytic Incorporation of Pyromycin. Photolysis experiments were performed by using ribosomes and subunits (80 A_{260} units/mL either in 5 mM $MgCl_2$ (60S subunits and ribosomes) or in 2 mM $MgCl_2$ (40S subunits) in a buffer of 50 mM triethanolamine, pH 7.4, and 50 mM KCl. Samples (140 μL, 2-mm solution depth) previously incubated with puromycin, either alone or in conjunction with other antibiotics (4 °C, 30 min), were irradiated at 4 °C for the periods of time indicated with a low-pressure mercury lamp having a maximum output at 253.7 nm and producing 2.9×10^2 erg mm^{-2} s^{-1} at the distance of the sample (10 cm). Incident radiation doses were determined by ferrioxalate actinometry (Parker, 1953). Immediately after photolysis, the mixtures were usually precipitated with 2 volumes of ethanol; the pellets were then extensively washed and tested for radioactivity after incubation with 1 mL of tissue solubilizer (2 h at 50 °C). During preliminary experiments, procedures such as ethanol or trichloroacetic acid precipitation (4 °C), dialysis, and sedimentation through a sucrose cushion were tested in order to find the best method of excluding the noncovalent sticking label.

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We observed that all methods gave the same results, except trichloroacetic acid precipitation which repeatedly gave lower incorporation values in the case of 40S subunits and 80S ribosomes, i.e., a loss of 52 and 20%. Loss was due almost exclusively to breaking of puromycin–protein bonds. Incorporation levels were calculated by assuming that 1 A_{260} unit equals 16.6, 24.6, and 51.0 pmol of ribosomes, 60S, and 40S subunits, respectively.

In order to locate incorporation sites, ribosomes and subunits irradiated and precipitated by ethanol were dissociated into their protein and RNA moieties using the Mg^{2+}–acetic acid procedure (Hardy et al., 1969). As will be seen, incorporated [^{3}H]puromycin was found in both the soluble (i.e., protein) and the insoluble (i.e., RNA) fractions (see Results). It may therefore be argued that the label found in the rRNA fractions is indirectly associated with rRNA through proteins linked to the RNA as a result of radiation. To check this latter possibility, we dissolved each labeled rRNA fraction (derived from 4 A_{260} units of subunits or ribosomes) in 1 mL of 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.1% sodium dodecyl sulfate ($NaDodSO_4$)[1] and divided the mixture into two identical samples. Ribonuclease-free Pronase (10 μg) was added to one, and both were incubated at 37 °C for 30 min (Abdurashidova et al., 1979). Immediately after incubation, the mixtures were precipitated with ethanol and counted. Occasionally, ribosomal RNAs extracted by phenol were submitted to one-dimensional gel electrophoresis using 3 and 2.4% acrylamide for 18S and 28S RNA, respectively (Loening, 1967). Protein fractions were dialyzed against acetic acid prior to being lyophilized and counted.

The concentration dependence of puromycin labeling was determined essentially as described by Coopermann et al. (1975) and Coopermann (1980). Ribosomes or isolated subunits were irradiated in the presence of a constant amount of radioactive puromycin with increasing amounts of nonradioactive antibiotic and then precipitated by ethanol. Experiments were carried out for a fixed length of time such that photoincorporation was proportional to the total incident light dose. As the effective dose was also inversely proportional to the absorbance of the mixture at 260 nm, we corrected the incorporation data to a constant effective dose by taking into account the contribution of puromycin absorbance to total absorbance of the UV-irradiated solutions. If the amount of [^{3}H]puromycin incorporated into a given particle reaches a saturation value as a function of ligand concentration, this strongly favors the hypothesis of a site-specific labeling. However, if the curve is neither a straight line nor a pure saturation curve, incorporation should result from two processes, one which saturates with respect to puromycin concentration (specific incorporation) and another which increases linearly with puromycin concentration (random incorporation). This model is described by

$$\frac{\text{pmol of puromycin incorporated}}{\text{pmol of particle}} = \frac{S[\text{puromycin}]}{K_d + [\text{puromycin}]} + L[\text{puromycin}]$$

where K_d is the apparent dissociation constant of the label for the specific site, S is the covalent labeling at saturation, and the slope (L) is a characteristic of the linear process. Linear contribution must be subtracted from the experimental values in order to obtain the hyperbolic saturation curve.

Polyacrylamide Gel Electrophoresis. Protein samples obtained by using the Mg^{2+}–acetic acid procedure were treated with iodoacetamide prior to analysis on one- and two-dimensional polyacrylamide gel electrophoresis, performed according to Madjar et al. (1979). At first, 2-D polyacrylamide gel electrophoresis in a basic–acidic system was used; thereafter, the first dimension of 2-D polyacrylamide gel electrophoresis was carried out by using either a basic or an acidic buffer system, pH 8.6 or 5.5, respectively. The 2-D and the 1-D polyacrylamide gel electrophoresis were carried out simultaneously in the presence of $NaDodSO_4$ (see Figure 3). Gel strips were sliced into approximately 2-mm slices, incubated with tissue solubilizer, and counted. The recovery of radioactivity was determined by summing across the gel and correcting for counting efficiency. The recovery from gel to gel was 43 ± 10%. In all the 2-D polyacrylamide gel electrophoresis experiments, stained protein spots were cut out and examined for the presence of radioactive puromycin. In our initial experiments, in order to detect all radioactive products, the remainder of the slab was cut up into pieces, and the pieces were counted. Thereafter, the procedure was reduced to the measurement of radioactivity in all the small pieces surrounding each of the stained proteins, with identical results. The recoveries from 2-D polyacrylamide gel electrophoresis were compared with one another and normalized to a 10% recovery.[2] Corrections were made for differences in the number of picomoles of subunits or ribosomes used and in the specific radioactivity of puromycin. The code used for numbering the proteins has already been reported and corresponds to that adopted recently as "uniform nomenclature" (McConkey et al., 1979; Madjar et al., 1979).

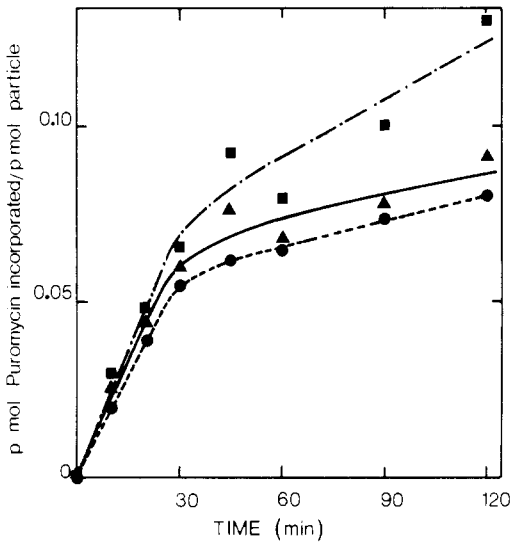

FIGURE 1: Kinetics of photoincorporation of puromycin into ribosomes and subunits. Ribosomes (▲) and either 40S (■) or 60S (●) subunits were irradiated with a 2.6-fold molar excess of [^{3}H]puromycin (6 μM, 5.7 Ci/mmol, 80 A_{260} units of ribosomes/mL) during increasing lengths of time (see Materials and Methods). Following irradiation, aliquots were precipitated with ethanol and tested for radioactivity (see Materials and Methods). The radioactivity found in control nonirradiated samples has been subtracted from the values given.

Results

Photoincorporation of Puromycin into Ribosomes and Their Constituents. Ribosomes were irradiated with [^{3}H]puromycin under the conditions and for the periods of time specified in Figure 1. Following irradiation, they were precipitated with ethanol and tested for incorporated radioactivity. A total of

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; 1-D and 2-D, one-dimensional and two-dimensional.

[2] The total radioactivity recovered from stained proteins was 10 ± 3% of that applied in the first dimension, which agrees with the yields reported by Grant et al. (1979a).

15–20% of the label recovered on irradiated mixtures was most likely in peptidylpuromycin, as it was found in nonirradiated samples.[3] Incorporation of label increased linearly for 30 min and then levelled out (Figure 1).

The incorporated radioactivity was mainly due to the covalent binding of [^{3}H]puromycin and not to a sticking of the irradiated product, since only small amounts of radioactivity were recovered when the antibiotic was irradiated alone and then added to nonirradiated ribosomes. Separately prephotolyzing either ribosomes (80 A_{260} units/mL, 30 min) or puromycin (2 μM, 30 min) prior to combining and photolyzing them together (30 min) gave incorporation values only 15–20% lower than those from experiments omitting the prephotolysis step.

In order to see which subunit had retained [^{3}H]puromycin, ribosomes incubated with a 5-fold molar excess of antibiotic were irradiated 30 min and then separated into ribosomal subunits as usual (see Materials and Methods). We observed that both subunits were labeled, the 60S subunit being labeled about twice as much as the 40S one. There was also a large loss of radioactivity and a smaller decrease of the 260-nm absorbance, which suggests that many ^{3}H-labeled nascent chains and nonribosomal proteins had been extensively eluted by 0.5 M KCl during preparation of subunits. This was supported by subsequent analysis of the proteins (see below).

Photoaddition of [^{3}H]puromycin to isolated 40S and 60S subunits was investigated, under the same conditions as those used with ribosomes (Figure 1). The same results were obtained for the different controls except, as was to be expected, a lower background level was found in unirradiated samples (about 2% of total incorporation). Incorporation of label into subunits also proceeded linearly for 30 min, with the same slope as that for ribosomes. Under prolonged irradiation, the rate of incorporation progressively decreased. Surprisingly, 40S subunits were found to retain significantly more puromycin than 60S subunits or ribosomes when irradiated longer than 30 min (Figure 1). A total of 18, 40, and 18%[4] of radioactive label associated to the 40S and 60S subunits and ribosomes was directly bound to rRNA as could be judged from the radioactivity recovered in pronase-treated RNA fractions (see Materials and Methods), the remainder being incorporated into the protein moieties. Proof that the label was covalently linked to 18S and 28S rRNA also came from analysis of phenol-extracted RNAs by gel electrophoresis in the presence of NaDodSO$_4$. Labeling of small rRNAs from large subunits (5–5.8 S), and of 18S and 28S RNAs from subunits prepared by using ribosomes labeled with [^{3}H]puromycin, was not determined. It is interesting to note that protein and RNA labeling within subunits increased linearly as a function of time, at least up to 30 min of irradiation. As a consequence of all photoincorporation kinetic results, the standard time of photolysis was set to 30 min in all the following experiments, as a compromise between the extent of labeling and the loss of biological activity in poly(U)-directed poly(phenylalanine) synthesis, at most–30% after 30 min of irradiation of either ribosomes or subunits (Reboud et al., 1978, 1980a; Buisson et al., 1979). Table I summarizes the results obtained as the ionic conditions of the labeling are modified. Thus, the labeling of both isolated subunits increases as Mg^{2+} concentration is reduced and KCl concentration is increased.

[3] Under our conditions (4 °C, low KCl buffer), peptidylpuromycin is not released (Blobel & Sabatini, 1971).

[4] The latter value is probably artificially lowered because ribosomes contain nascent chains and extraneous proteins which are also labeled (see below).

Table I: Effect of Ionic Conditions on the Labeling Reactions

ribosomal preparation	[$MgCl_2$] (mM)	[KCl] (mM)	puromycin incorporated (cpm)
60S	5	50	3953
	5	350	8587
	5	500	8048
	1	50	7550
40S	2	50	5046
	2	350	11181
	1	50	9082

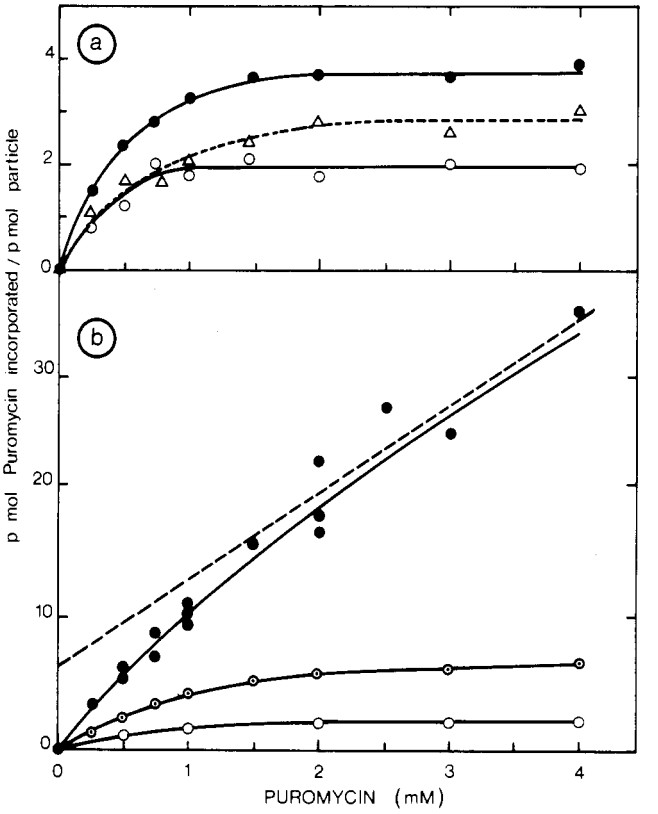

FIGURE 2: Concentration dependence of photoincorporation of puromycin into ribosomes, isolated 60S subunits, and their derived protein and RNA moieties. Experimental conditions were 80 A_{260} units/mL, 5.7 Ci/mmol [^{3}H]puromycin, 30-min irradiation time, and 0.025-mL assay volume (see also Materials and Methods). Following irradiation and precipitation by ethanol, aliquots of ribosomes and subunits were dissociated into their protein and RNA moieties, and the radioactivities were counted after RNA fractions had been incubated with Pronase (see Materials and Methods). (a) Saturation curves of 60S subunits (●), P60S protein (△), and rRNA fraction (○). (b) Saturation curve of crude ribosomes: experimental incorporation values (●), data obtained from the previous curve from which the linear contribution (broken line) has been subtracted (see Materials and Methods) (⊙), and saturation curve of rRNAs (○).

These results are probably related to the increase of the monomer species observed under either low Mg^{2+} or high KCl concentrations (sedimentation patterns not shown). However, such low Mg^{2+} and high KCl concentrations were not retained for they proved to have a destabilizing effect on the ribosomal structure during irradiation.

Concentration Dependence of Puromycin Labeling. In two independent experiments, labeling of 60S subunits appeared to be site specific as did labeling of their RNA- and protein-derived fractions (Figure 2a). Similar saturation curves were obtained by using either isolated 40S subunits or their derived RNA and protein fractions. However, the experimental values corresponding to 40S subunit labeling were more dispersed, and the values at saturation were about 2 times higher for 40S

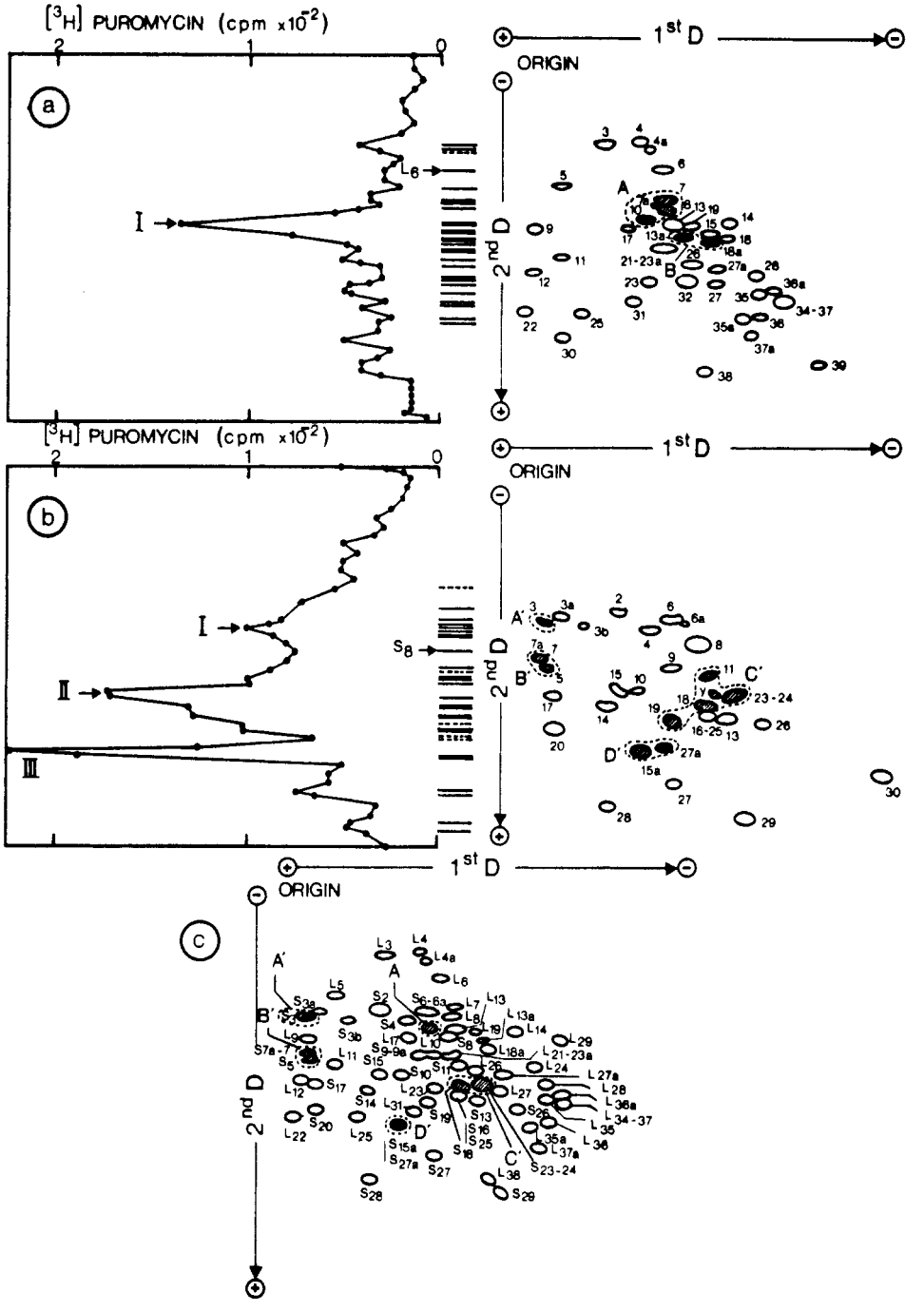

FIGURE 3: Polyacrylamide gel patterns of proteins extracted from either isolated subunits or crude ribosomes labeled with [^{3}H]puromycin. 1-D and 2-D electrophoretograms (in $NaDodSO_4$ and basic $NaDodSO_4$ systems, respectively) of P60S and P40S proteins from isolated subunits are shown on the right of panels a and b. Only 2-D polyacrylamide gel electrophoresis has been reported in the case of ribosomes (c, see text). Reported counts per minute, at the left of the panels a and b correspond to proteins from 100 pmol of particles, analyzed in 1-D polyacrylamide gel electrophoresis. Major labeled proteins are indicated by hatched spots in 2-D polyacrylamide gel electrophoresis. Experimental conditions: 80 A_{260} units/mL; 30-min irradiation time; puromycin in a 5:1 molar excess over particles.

subunits and their derived proteins than those determined by using 60S subunits and P60S proteins. The incorporation into crude ribosomes resulted from two processes: one which saturated with respect to puromycin concentration and one which increased linearly with puromycin concentration with a slope (L) of 6.5 mM^{-1} (see Materials and Methods, Figure 2b). Values of the apparent dissociation constant K_d calculated from all these data were 0.3, 0.5, and 0.7 mM for 60S subunit, 40S subunit, and ribosome labeling with puromycin, respectively. It is possible that the labeling values at saturation are overestimated, since, as reported by Pellegrini & Cantor (1977) in similar photolabeling experiments, only part of the radioactivity was recovered after extraction of proteins and RNA and their analysis by polyacrylamide gel electrophoresis.

Locating Protein Incorporation Sites. In order to maximize the proportion of specific labeling, very low concentrations of puromycin were used, i.e., a 0.4–5 molar ratio of puromycin over ribosomes. Identification problems raised by the possibility of displacement of labeled proteins relative to unlabeled ones on the electrophoretograms were solved by analyzing the same samples of ribosomal proteins simultaneously in 1-D and 2-D polyacrylamide gel electrophoresis (Figure 3a–c). This method has already been shown to be appropriate for precise identification of labeled products in affinity labeling experiments (Pellegrini & Cantor, 1977). Indeed, although ribosomal proteins are not well resolved in 1-D polyacrylamide

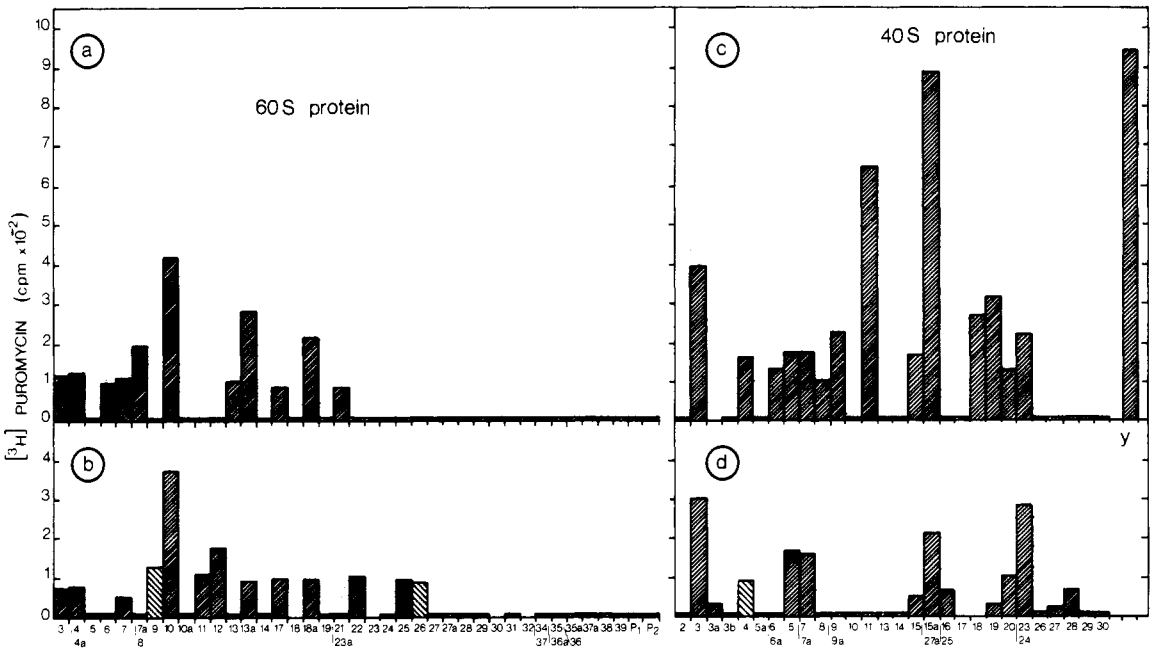

FIGURE 4: Distribution of [^{3}H]puromycin over the proteins from 60S and 40S ribosomal subunits either used in the free state (a and c) or associated within ribosomes (b and d). Proteins have been resolved by 2-D polyacrylamide gel electrophoresis using either the basic NaDodSO$_4$ system (see Figure 3) or the acidic NaDodSO$_4$ system (only for proteins L10a, P1, P2, S3a, and S5). Counts reported are relative to the areas which correspond as closely as possible to the stained protein spots. The average radioactivity (30 cpm) found outside the stained spots and region Sy has been subtracted from the values given. There is reason to believe that the radioactivity found in some proteins from ribosomes [L9, L26, and S4 (▨)] is due to contamination from S5–S7–7a, S23–24, and L10, respectively, because of poor resolution of these proteins. Values are for proteins recovered from 1 nmol of either subunits or ribosomes and are normalized to a 10% recovery (see Materials and Methods). Each value is the average obtained from three separate experiments; 0.35–1 mg of labeled proteins was analyzed per experiment, using five 2-D polyacrylamide gel electrophoresis plates.

gel electrophoresis, labeled products are expected to be less shifted than in two-dimensional gels. The radioactivity of each band in 1-D polyacrylamide gel electrophoresis was compared quantitatively with that of the corresponding radioactive spots in 2-D polyacrylamide gel electrophoresis, taking into account the difference of recovery in the two systems. We observed that the staining intensity of irradiated samples in either 1-D or 2-D polyacrylamide gel electrophoresis was identical with that observed by using control unirradiated samples, which suggests that under our conditions of irradiation time and material concentration, no protein–RNA cross-links have been formed. This conclusion would also agree with the results of RNA labeling showing that it withstood Pronase treatment almost completely.

When proteins from isolated 60S subunits were analyzed in 1-D polyacrylamide gel electrophoresis only one prominent peak (I) of radioactivity was observed, which migrated 1.4 times farther than protein L6, the only well-resolved protein in this system. Two-dimensional electrophoretograms in a basic NaDodSO$_4$ system showed that two very close radioactive areas fell in the region of the gel corresponding to peak I. The most radioactive gel section (A) contained the major labeled protein L10 and proteins L7a–8 labeled to a lesser extent. A smaller amount of radioactivity was recovered from region B, which contained proteins L13a and L18a. The remaining radioactivity was spread in small amounts over eight other proteins among the 37 isolated (Figures 3a and 4a). No radioactivity was detected in the region corresponding to the phosphoproteins P1 and P2.

The one-dimensional polyacrylamide gel pattern of proteins from puromycin-labeled 40S subunits showed three peaks of radioactivity (I, II, and III) whose relative size from one to another varied according to the experiments (Figure 3b). The results of two-dimensional gel electrophoretograms (Figures 3b and 4c) showed that there were three main radioactive areas (A′, C′, D′) which corresponded almost quantitatively to the three peaks described above and whose radioactivity could be ascribed respectively to proteins S3, S11, S18, S19, S23–24, and S15a–27a. Labeling dominated in the region (y) centered slightly to the left of S23–24, above S18, which indicated that one labeled protein, difficult to identify in our conditions as S23–24 or S18, had a slightly altered electrophoretic mobility, and might then contaminate S11. Some radioactivity was also found in region B′ which contains S5 and S7–7a.[5] No radioactivity was found in the region corresponding to the acidic proteins S12 and S21.

In contrast to the patterns obtained by using proteins derived from isolated subunits, the 1-D polyacrylamide gel electrophoresis of ribosomal proteins from puromycin-labeled ribosomes yielded a pattern showing a wide distribution of radioactivity. In particular, we found, reproducibly, that the region of very high molecular weight, which is hardly stained for proteins, contained 32–40% of the total radioactivity recovered. Protein aggregation through S–S bridges was unlikely, since protein samples were treated with iodoacetamide prior to analysis. We therefore assume that this spreading of radioactivity was due to photoincorporation of puromycin into nascent chains and contaminant nonribosomal proteins. For this reason, 1-D polyacrylamide gel electrophoresis data, which are meaningless, are not given. Radioactivity was found in five regions of the 2-D electrophoretograms (basic NaDodSO$_4$ system, Figure 3c) corresponding to those already observed with isolated subunits, i.e., A (protein L10), A′ (protein S3), B′ (proteins S5 and S7–7a, which may contaminate surrounding protein L9), C′ (proteins S23–24 and/or S18, which may contaminate L26 slightly), and D′ (proteins S15a–27a). From our gel results (Figures 3c and 4), we calculated that

[5] Since these proteins are not completely separated from one another, there is no certainty as to whether only one is labeled or both.

some of the major reaction products, such as proteins L10 and S3 and possibly S23–24,[6] were labeled to a similar extent whether the target was a crude ribosome or an isolated (60S or 40S) subunit. In contrast, other major labeled components in isolated subunits, and in particular in 40S subunits (S11, S15a–27a, S18, and S19), were almost totally unreactive when included in ribosomes. It can be estimated that for almost the same number of proteins identified, labeling of the total P40S proteins was reduced by about two-thirds in crude ribosomes as compared with that recovered from free subunits[7] (Figure 4c,d). In contrast to this, total labeling of P60 proteins was little changed whether subunits were free or in ribosomes (Figure 4a,b). No attempts were made to determine which proteins were labeled in 40S–60S equimolecular mixtures since we know that several different types of couples between 40 S and 60S are formed under these conditions.

Competition Experiments. In order to distinguish, among the labeled proteins, the products resulting from the modification of a functional site from those of any eventual nonspecific reaction, competitive experiments were carried out. We first considered the possibility of trying to inhibit the labeling of 60S and 40S subunits with an excess of deacylated tRNA and aminoacyl-tRNA, respectively. The rationale for using nonacylated tRNA was that it is assumed to bind to a functional site (P site) of the 60S subunits, while poly(U)-dependent binding of Phe-tRNA occurs on a site of the 40S subunits which belongs to the A site. However, comparison with our earlier experiments would have been very difficult since tRNA binding requires 13–20 mM Mg^{2+} concentrations, which are far higher than those used in our experiments, and this can affect puromycin incorporation (see Table I). Moreover, solutions of very different absorbance would have had to be irradiated. We therefore undertook competition experiments with two types of antibiotics: first, gougerotin and amicetin, which both inhibit peptide bond formation and act on the large subunits at the peptidyltransferase center on the acceptor site, in a certain part which is common to bacterial and eukaryotic ribosomes (Carrasco et al., 1976); second, tetracycline, which blocks protein synthesis in bacterial and mammalian systems and interacts with the smaller and the larger ribosomal subunits. It is not yet clear which type of interaction is more relevant for the inhibitory effect of tetracycline on protein synthesis (Vazquez, 1978). Experiments with these antibiotics can be made under similar conditions of absorbance and Mg^{2+} concentration as those used with puromycin (Barbacid & Vazquez, 1974). Figure 5 shows the variation of [^{3}H]puromycin incorporation into one-dimensional gel electrophoresis peaks, as the molar ratio of the antibiotic competitor over ribosomes varied. We preferred to use the results of one-dimensional gel analysis rather than those of two-dimensional gels because of their better reproducibility. As can be seen, gougerotin, amicetin, and tetracycline compete with [^{3}H]puromycin for the 60S proteins located in the major radioactive peak (I) (Figure 5a). These data suggest that gougerotin and amicetin, and also tetracycline, bind to the same site as puromycin on 60S subunits. On the contrary, the radioactivity of the major labeled fraction of 40S subunit proteins (peak II) showed practically no variation whatever the competitor concentration was. Similar observations were made with the two other labeled fractions (peaks I and III), except in the presence of a 50-fold molar excess of gougerotin. Only, under these conditions, a significant decrease in the radioactivities of peaks I and III was observed.

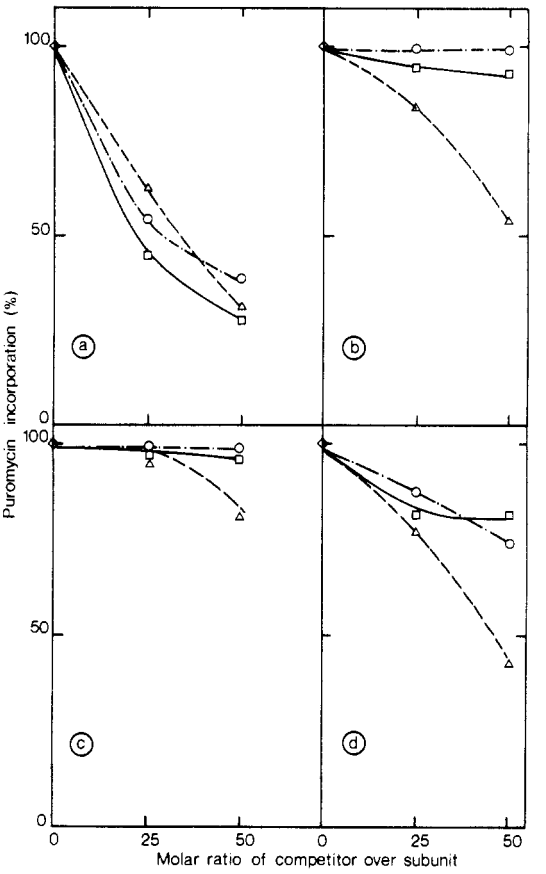

FIGURE 5: Incorporation of puromycin into the major radioactive peaks seen in one-dimensional 60S and 40S protein gels as a function of the molar ratio of antibiotic competitor over ribosomal subunits. (a) Variation of radioactivity of peak I from 60S protein gels as shown in Figure 3a. (b, c, and d) Variation of radioactivity of peaks I, II, and III, respectively, from 40S protein gels as shown in Figure 3b. Experimental conditions: puromycin alone (◇), same as described in Figure 3 and 4; plus gougerotin (△); plus amicetin (○); plus tetracycline (□). The results have been scaled so that the peak with maximum radioactivity in each case is assigned a value of 100. In (a), (b), (c), and (d), the maximum radioactivity was respectively 430, 574, 1000, and 640 cpm.

Discussion

Puromycin was induced to incorporate covalently into rat liver ribosomes and subunits on direct UV irradiation. Until now, this technique had been applied successfully only using bacterial ribosomes. In a recent study, photochemical reaction of unmodified puromycin with mammalian ribosome components was not observed, but the irradiation conditions were different from those used in our experiments (Böhm et al., 1979).

We have no direct evidence that the sites specifically labeled on 60S subunits and on ribosomes belong to the functional one(s). The apparent dissociation constant, K_d, values for 60S subunits and ribosomes are similar to that found for *E. coli* ribosomes, which is of the same order of magnitude as the K_m value reported for peptidylpuromycin or fragment assays (Coopermann et al., 1975; Coopermann, 1980). They are, however, much higher than the K_m value determined in the acetylphenylalanylpuromycin assay by Thompson & Moldave (1974), using rat liver ribosomes. In any case, these K_d and K_m values should be compared with caution since the assays

[6] Definitive identification of these proteins is difficult since they migrate in a protein-dense region of the gel.

[7] This result cannot be related to a lowered puromycin/ribosome ratio in ribosome experiments, because of the relatively small amount of puromycin incorporated into the nascent chain, since this would also have affected similarly labeling of the P60S proteins, which was not observed.

are performed with very different buffers: the peptidylpuromycin assay necessitates very high monovalent cation concentrations and alcohol, while photoincorporation of puromycin does not. However, in the case of 60S subunits, there are strong arguments which favor the hypothesis that covalent puromycin binding occurs on a specific and functional site: the relatively small distribution of label among the ribosomal proteins and the diminution of the labeling reaction when using antibiotics such as gougerotin and amicetin known to act at the peptidyltransferase center specifically. The results of our competition experiments with tetracycline were recently confirmed by experiments which showed that [^{3}H]tetracycline was directly photoincorporated into those proteins (L10, L13a, and L18a) which were found to be at the puromycin-binding sites, either on ribosomes or on isolated 60S subunits, protein L10 being the most labeled one.[8] Our recent findings that on isolated 60S subunits protein L10 was protected against salt removal by prior fixation of deacylated tRNA at the P site (Reboud et al., 1980b) and directly photoreacted with deacylated [^{32}P]tRNA when at the P site[8] support the tentative conclusion that, among the [^{3}H]puromycin-labeled 60S subunit proteins, at least one, protein L10, forms part of a functional site. This protein was also found by other authors to react, in affinity labeling experiments, with either photoactivable puromycin or aminoacyl-tRNA derivatives (Böhm et al., 1979; Czernilofsky et al., 1977; Stahl et al., 1979). Such an agreement about protein L10 is observed in spite of the fact that the methods used for ribosome preparations differed from ours: all the above authors employ puromycin treatment and high salt washing to release nascent protein while we do not (see Materials and Methods). Such a result suggests that puromycin-binding sites on L10 are quite insensitive whether crude ribosomes mainly consisting of polysomes, washed 80S ribosomes, or isolated subunits are the target.

In the case of 40S subunits, the higher dispersion of the radioactivity measurements, the wide distribution of label among ribosomal proteins, and the variability of the relative radioactivities of peaks I, II, and III seen in 1-D electrophoretograms suggest that the observed covalent binding is not restricted to specific binding sites. However, the negative results of competition experiments using amicetin and gougerotin are not conclusive, since these antibiotics are known to act on 60S subunits (Barbacid & Vazquez, 1974). The absence of competition with tetracycline is more significant, since this compound does bind to 40S subunits. We recently confirmed that [^{3}H]tetracycline binds to 40S subunit proteins different from those which were found to interact with puromycin.[8] Alternative hypotheses can be proposed to explain, at least partly, such a wide distribution of the label among 40S subunit proteins: (1) a high heterogeneity of 40S subunits so that some of them are in different states and therefore are not labeled exactly to the same extent, within the same proteins, and indeed such a heterogeneity of the 40S subunits has been recently observed towards high salt treatment[8] while 60S subunits yielded homogeneous species under the same conditions (Reboud et al., 1980b); (2) a proximity of the labeled 40S subunit proteins, as evidenced by Terao et al. (1980), for proteins S3–S11, S5–S7–7a, S7–S18, and S23–24, using free subunits and a bifunctional cross-linking reagent; (3) finally, it is worth considering the observations made by Pellegrini & Cantor (1977) that, in labeling studies, isolated *E. coli* subunits showed a higher level of nonspecific reaction than intact ribosomes. In our experiments, if one considers just the results obtained with crude 80S ribosomes, the conclusions concerning protein specificity remain unchanged for L10 whereas for 40S subunit proteins they are restricted to protein S3 and possibly S23–24. In any case, this suggests that puromycin-binding sites are located on both subunits and, moreover, not at their interface. In this context, it is interesting to note that the elongated protein S3 [which corresponds to S2 in the nomenclature of Welfle et al. (1972)] has been located by immune electron microscopy in the region between the head and the body of the 40S subunit, on the side opposite to the protuberance, i.e., the side which is not in contact with the large subunit (Lutsch et al., 1979). With regard to its function, S3, as L10, has been located at or near the P site, since it was involved in eIF-2 Met-tRNA$_f$ binding (Noll et al., 1978) and could be cross-linked to this initiation factor (Westermann et al., 1979). This implies that puromycin can react with components of the A and also of the P site. These sites are located on both subunits, very close to each other. Moreover, protein L10 is easily removable by salt (Reboud et al., 1980b) whereas S3 is not,[8] and both of them associate to ribosomal particles at later stages of the maturation process (Auger-Buendia et al., 1979).

Among the few affinity labeling studies carried out on mammalian ribosomes using derivatives of puromycin or tRNA, which we have already mentioned, only one showed a slight labeling of 40S subunit proteins S3 and S26 (Böhm et al., 1979). None of these studies mentioned that rRNAs were labeled, but the reactions were directed essentially toward protein components. The results of our experiments, in which a photochemical reaction with unmodified puromycin was used, recall those obtained with *E. coli* ribosomes which retain the antibiotic on the proteins of the large but also of the small subunit and on rRNAs (Coopermann et al., 1975; Coopermann, 1980; Jaynes et al., 1978).

[8] A. M. Reboud and S. Dubost, unpublished results.

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Effect of Vitamin D Deficiency on in Vitro Labeling of Chick Intestinal Proteins: Analysis by Two-Dimensional Electrophoresis†

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ABSTRACT: Duodenal tissue from vitamin D_3 replete chicks was labeled in vitro for 2.5 h with ^3H-labeled amino acids and then combined with tissue from severely vitamin D deficient chicks incubated in an identical fashion with ^{14}C-labeled amino acids. Four double-labeled samples thus obtained were separated into pellet and cytosol fractions by centrifugation, the acidic proteins in each fraction separated by two-dimensional electrophoresis, and the separated proteins analyzed for changes in $^3H/^{14}C$ ratio by the McConkey method of double-label autoradiography. The $^3H/^{14}C$ ratios for proteins with high isotope incorporation were then determined by direct measurement of radioactivity. Of the 100 proteins resolved in the pellet fraction, two were found with $^3H/^{14}C$ ratios greater than base line. Protein 1 (M_r 27 000) was identified by comigration as membrane-associated, vitamin D induced calcium binding protein. Protein 23 (M_r 76 000 and unknown identity) was increased in $^3H/^{14}C$ ratio by 2.7-, 2.0-, 2.8-, and 1.4-fold over base line in samples I–IV, respectively; this protein stained very faintly with Coomassie blue. The $^3H/^{14}C$ ratio of β- and γ-actin was low in each sample, being 0.61, 0.54, 0.28, and 0.67 of the base-line ratio in samples I–IV, respectively. In the cytosolic fractions, the calcium binding protein was found to have a $^3H/^{14}C$ ratio approaching infinity in each sample. In addition, an unidentified cytosolic protein (M_r ~76 000) was found to be enriched 1.5-, 2.2-, 4.9-, and 1.7-fold over the base-line ratio. This protein also stained faintly with Coomassie blue. The observed deviations from the $^3H/^{14}C$ base-line ratios probably reflect differences in rates of protein synthesis brought about either directly by 1,25-dihydroxyvitamin D_3 or indirectly via changes in serum calcium or other serum factors.

The most pronounced physiological action of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] is to stimulate directly transcellular calcium transport across intestinal villi. Although several theories have been postulated concerning the mechanism of this stimulation (Zerwekh et al., 1976; Wilson & Lawson, 1977; Bickle et al., 1978; Lane & Lawson, 1978; Rasmussen et al., 1979; MacLaughlin et al., 1980), the precise cellular and molecular scheme of events leading to increased calcium transport in intestine is unknown.

One reasonable hypothesis (Zerwekh et al., 1976) is that since 1,25-$(OH)_2D_3$ is steroidlike in structure, possesses high-affinity intestinal receptors, and localizes in intestinal nuclei (Stumpf et al., 1979), its mechanism is similar to that of other steroid hormones, i.e., it acts via stimulation of transcription of specific genes (Gorski & Gannon, 1976). The vitamin D dependent calcium binding protein (CaBP) is one such gene product (Wasserman et al., 1978); however, the time

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[1] Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CaBP, calcium binding protein; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole.

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