RAPID DETERMINATION OF THE SPECIFIC RADIOACTIVITIES OF RNA PRECURSOR POOLS (UTP AND ATP) BY AN ENZYMIC METHOD

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1. Introduction

Incorporation of exogenous nucleosides is commonly used as a measure for net RNA or DNA formation. However, this is valid only in short term experiments and in the absence of pool size alterations of the nucleoside triphosphate in question. Thus, a drastic decrease in UTP concentration with a concomitant increase in specific radioactivities of UTP and RNA has been observed in HeLa cultures treated with exogenous cAMP in spite of an inhibition of net RNA formation [1]. Furthermore, in labeling experiments exceeding 30 min incubation with the nucleosides the somewhat delayed conversion of UTP to CTP and of ATP to GTP will introduce an additional source of error [2,3]. The correlation of precursor pool specific radioactivities with the labeling of nucleic acids, then, appears to be essential when the correct information on the synthetic pattern of macromolecules is to be obtained. Similarly, UTP specific radioactivity proved to be an essential parameter for the exact determination of mRNA half-life in mammalian cells (cf. [4]). Useful methods are therefore required to evaluate eventual changes in precursor pool size and labeling. This paper describes the determination of ATP specific radioactivity and the improved method for an analogous analysis of UTP (cf. [5]) using 'enzymic displacement' for selective quantitation of the radioactive label.

2. Materials and methods

[5-^{3}H] uridine (spec. act. 25 Ci/mmole), [5-^{3}H] uridine triphosphate (spec. act. 15 Ci/mmole), [2-^{3}H] adenosine (spec. act. 9 Ci/mmole), [2-^{3}H] adenosine triphosphate (spec. act. 19 Ci/mmole) were purchased from Buchler–Amersham, Braunschweig; modified Joklik medium (F-13) from Grand Island Biological Comp. USA; Nucleotides, nucleosides, bases, cofactors and enzymes were obtained from Boehringer, Mannheim, and from E. Merck, Darmstadt. Polyethylene thin layer plates (PEI cellulose) were obtained from Machery and Nagel, Düren (polygram Cel 300 PEI/UV_{254}) and from E. Merck, Darmstadt (DC-Plastikfolien, PEI cellulose F).

HeLa S3 cells were grown in suspension cultures as described previously [6].

2.1. *Acid soluble fraction*

An aliquot of the cell suspension (3×10^7 cells) was centrifuged at 1200 *g* for 5 min. The pellet was extracted with 1 ml ice-cold 0.4 N $HClO_4$ and kept in ice for 15 min. After centrifugation the supernatant was neutralized by the addition of 0.5 ml of 0.72 N KOH–0.16 N $KHCO_3$ and kept in ice for 15 min. $KClO_4$ was removed by centrifugation and the resulting supernatant was used for enzymic determination of the nucleotides.

2.2. *Base composition and base labeling in RNA*

Base composition and base labeling in RNA were analyzed in the acid insoluble fraction. The residue obtained after centrifugation of the $HClO_4$ extract was washed twice with 0.4 N $HClO_4$ and hydrolyzed with 0.5 N KOH at 25°C for 18 hr. After precipitation of DNA and proteins with $HClO_4$, the supernatant obtained by centrifugation was analyzed using electrophoretic separation of nucleotides at pH 3.5 as described previously [2].

2.3. *UTP concentration*

UTP was determined by the highly specific enzymic method of Keppler et al. [7] as adapted and described by Kramer et al. [5]: The extract of 4×10^6 cells (= 200 µl neutralized acid soluble fraction) was usually analyzed.

2.4. *ATP concentration*

ATP was determined by the hexokinase method [8] with slight modifications. In a total vol of 1.0 ml, 64,6 µmoles triethanolamine buffer pH 7.5; 0.33 µmoles NADP; 6.66 µmoles $MgCl_2$; 0.5 µmoles glucose; ± 13.3 µg hexokinase and 3.3 µg glucose-6-phosphate-dehydrogenase and 200 µl neutralized acid soluble extract (4×10^6 cells) were incubated at 25°C for 15 min and analyzed for increase in absorbance at 340 nm.

2.5. *Selective determination of nucleotide labeling by enzymic displacement*

2.5.1. UTP

50 µl of the neutralized acid soluble fraction were incubated at 37°C for 30 min in a total volume of 100 µl containing 15.4 µmoles glycine-KOH buffer pH 9.5; 192 nmoles EDTA; 576 nmoles ATP; 384 nmoles glucose-1-phosphate; 1.92 µmoles $MgCl_2$; ± 0.6 µg UDPG pyrophosphorylase. After incubation 5–50 µl of the mixture were applied to polyethylene thin layer plates (PEI/UV_{254}, MN 300, Machery u. Nagel, Düren)*, 3 cm from the lower edge together with 5 nmoles each of unlabeled markers (UTP, UDP, UMP, UDPG, uridine), and separated by one-dimensional chromatography according to Randerath [9], with slight modifications: After a preliminary ascending development with methanol–water (4:1, v/v) in order to remove salts and other interfering substances, the plates were dried in a cold stream of air and developed in 0.1 M LiCl until the solvent front had passed the origin by 1 cm. They were then transferred to 1.0 M LiCl until the solvent front had moved to 13 cm from the origin. The plates were dried, and sectioned into 10 or 5 mm × 22 mm strips according to the UV spots. The sections were scraped off with a razor blade and transferred into scintillation counting vials. 500 µl of 1.0 N HCl were added, and radioactivity was determined after the addition of 15 ml of dioxane scintillation mixture. The radioactivity (cpm) disappearing from UTP and appearing at the UDPG position in the samples with UDPG pyrophosphorylase represents the radioactivity of UTP in the acid soluble fraction.

For the determination of radioactivity, the layer section was transferred into a small centrifuge tube, extracted with 500 µl 1 N HCl, centrifuged, and an aliquot analyzed for dpm in a computer-aided liquid scintillation counter (BF 5000, Berthold und Friesecke – Diehl Algotronic).

2.5.2. ATP

40 µl of the acid soluble fraction were incubated for 30 min at 37°C in a total vol of 145 µl containing 6.48 µmoles triethanolamine buffer pH 7.5; 67 nmoles $MgCl_2$ 100 nmoles glucose**, ± 0.91 µg hexokinase. Chromatographic analysis of the nucleotides was performed as described for UTP, after the addition of 5 nmoles each of ATP, ADP, and AMP. The radioactivity disappearing from ATP and appearing at ADP position in the sample with hexokinase represents the radioactivity of ATP in the acid soluble fraction. Specific radioactivity (cpm/nmole) was calculated from the displaced radioactivity and the amount of nucleotide in the same aliquot.

Uridine and adenosine contents in the medium were analyzed as described previously [2].

3. Results and discussion

3.1. *Determination of UTP specific radioactivity*

Evaluation of the specific radioactivity of a metabolite requires selectivity in the determination of amount (µmoles) as well as of radioactivity (cpm). While in many cases specific methods for quantitation of amounts are available, determination of radio-

* Similar results were obtained with thin layer plates from E. Merck (DC-Plastikfolien, PEI-Cellulose F), when the ionic strength was reduced to 0.8 M. Each brand requires a test for optimal salt concentrations.

** Higher concentrations of glucose lead to a steadily increasing extinction with no clear end-point.

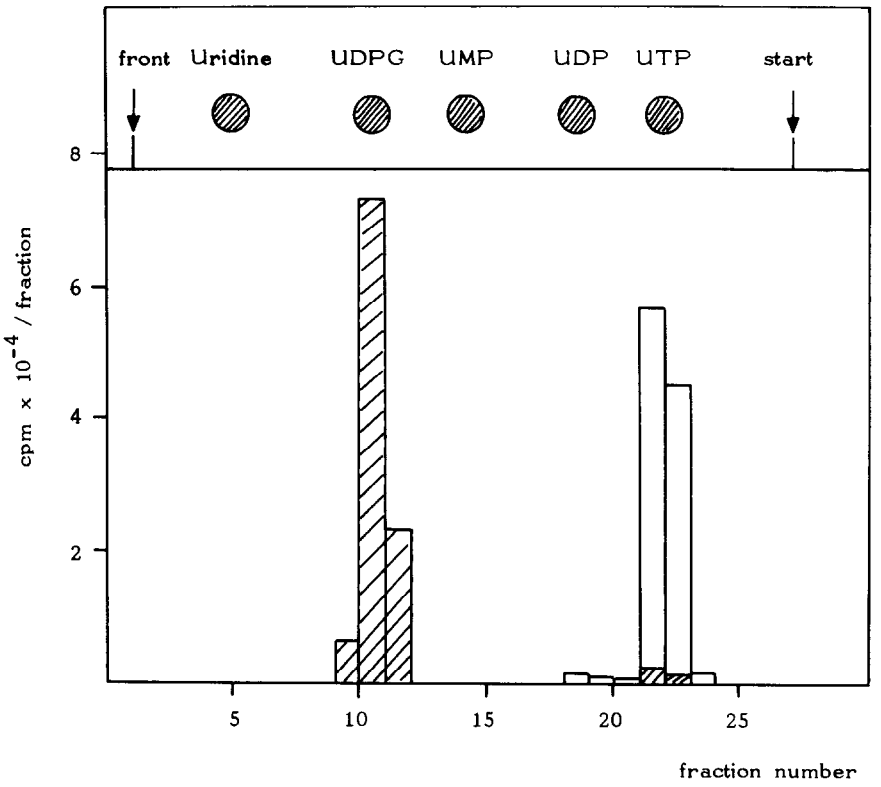

Fig. 1. 'Enzymic displacement' of UTP radioactivity to the UDPG position. 0.5 μCi [^{3}H] UTP (= 33 pmoles) were incubated in presence of non-labeled HeLa cell extract, containing 1.2 nmoles UTP, ± 0.6 μg UDPG-pyrophosphorylase for 30 min at 37°C. 20 μl of the incubation mixture were separated on PEI cellulose chromatography. For experimental details see Materials and methods. (shaded columns = + UDPG-pyrophosphorylase). The upper part indicates the position of the UV markers.

activity of a given metabolite in the presence of other labeled intermediates usually requires extensive purification. Kuhn and Hilz have introduced the 'enzymic displacement' as a method for a rapid and selective analysis of adenine label in NAD in cells exposed to [^{14}C] adenosine [10]: Extracts treated ± NAD glycohydrolase (or phosphodiesterase) were chromatographed and the displacement of cpm from the NAD position to the position of the enzymic reaction product was analyzed. In an analogous way, UTP label in cells exposed to [^{3}H] uridine could be determined selectively by treatment of acid-soluble extracts with UDPG-pyrophosphorylase (and Gl-1-P) thus displacing UTP radioactivity to the UDPG position and a subsequent electrophoretic separation [5].

Evaluation of UTP label has now been improved greatly with respect to speed and precision by thin-layer chromatography of uridine nucleotides on PEI cellulose. As shown in fig. 1, [^{3}H] UTP in the presence of added, non-labeled HeLa cell extract was displaced by incubation with UDPG-pyrophosphorylase and Gl-1-P to the UDPG position. Radioactivity disappearing at UTP and appearing at UDPG position was identical within 5% of error. Without enzyme (open area) about 98% of the radioactivity was found in the UTP position. After incubation (shaded area) nearly all radioactivity had been converted to UDPG. About 3% of the initial radioactivity remained at the UTP position. When the [^{3}H] UTP applied in this test was chromatographed on DEAE-cellulose thin-layer with 0.06 N HCl as solvent, a system to separate nucleoside triphosphates [11], only 97% of the radioactivity migrated with authentic UTP (not shown). To test the quantitative conversion

of UTP under the conditions described in more detail, [^{3}H] UTP was incubated together with increasing amounts of non-labeled UTP. Fig. 2 demonstrates that the reaction was completed within the first 20 min of incubation. Even at a 285-fold excess of 'cold' UTP (a total of 20 nmoles) the UV spot at the UTP position had completely disappeared within the first 20 min of incubation. Here again, the same amount of radioactivity at the UTP position strongly indicating the presence of a labeled compound not identical with UTP. This is further supported by the observation that increasing concentrations of enzyme did also not alter these values (not shown).

3.2. *Determination of ATP specific radioactivity*

ATP specific radioactivity could be determined in a way analogous to UTP: ATP was analyzed using hexokinase as a specific enzyme converting ATP to ADP in the presence of glucose and Mg^{2+}. When supplemented with glucose-6-phosphate dehydrogenase and NADP, $NADPH_2$ formation could be evaluated as a measure of ATP concentration [8]. The enzymic displacement of ATP label to the ADP position was achieved again with the hexokinase reaction and subsequent thin-layer chromatography of PEI cellulose Fig. 3 shows the chromatograms of [^{3}H] ATP in the presence of non-labeled acid soluble cell extract ± incubation with hexokinase. Most of the radioactivity (98%) was transferred from ATP to ADP position. About 2% remained at the ATP spot. A kinetic analysis of the ATP displacement (fig. 4) at two different concentrations of ATP revealed completion of the reaction within 30 min (37°C). Here too, the UV spot had disappeared completely from the ATP position and had moved to the ADP position within the first 20 min, while the residual cpm (~ 2% of total) remained at the original position, indicating the presence of a contaminating nucleotide not being displaced by the enzyme.

3.3. *Application of the methods for RNA precursor pool analysis*

The methods described were applied to follow the intracellular labeling of UTP or ATP during incubation

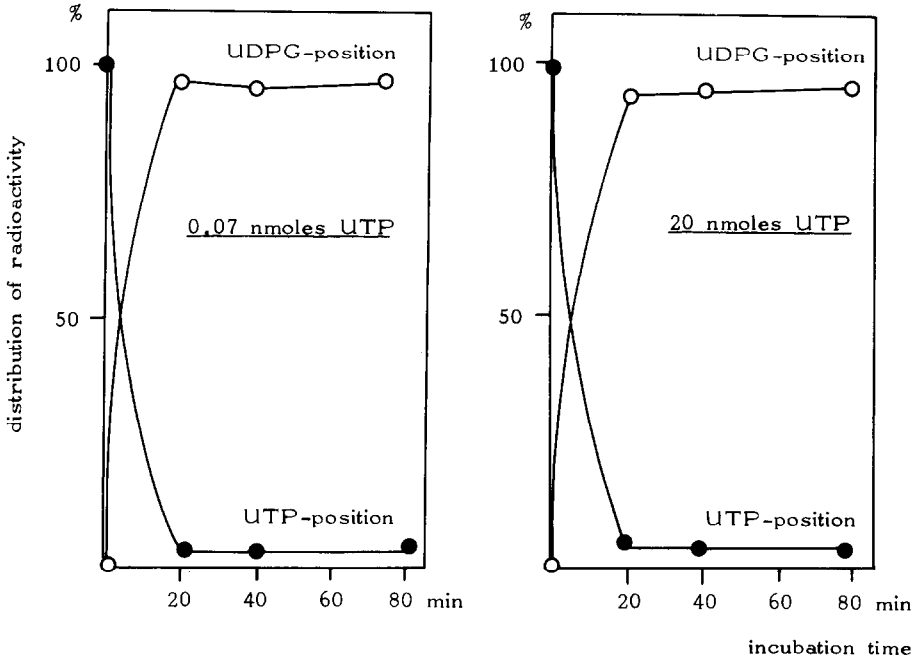

Fig. 2. Kinetic analysis of UTP displacement at two different UTP concentrations. [^{3}H] UTP ± added non-labeled UTP at the indicated concentrations were incubated with 0.6 μg UDPG-pyrophosphorylase at 37°C. At the times indicated 20 μl of the incubation mixture were separated on PEI cellulose chromatography as described in Materials and methods.

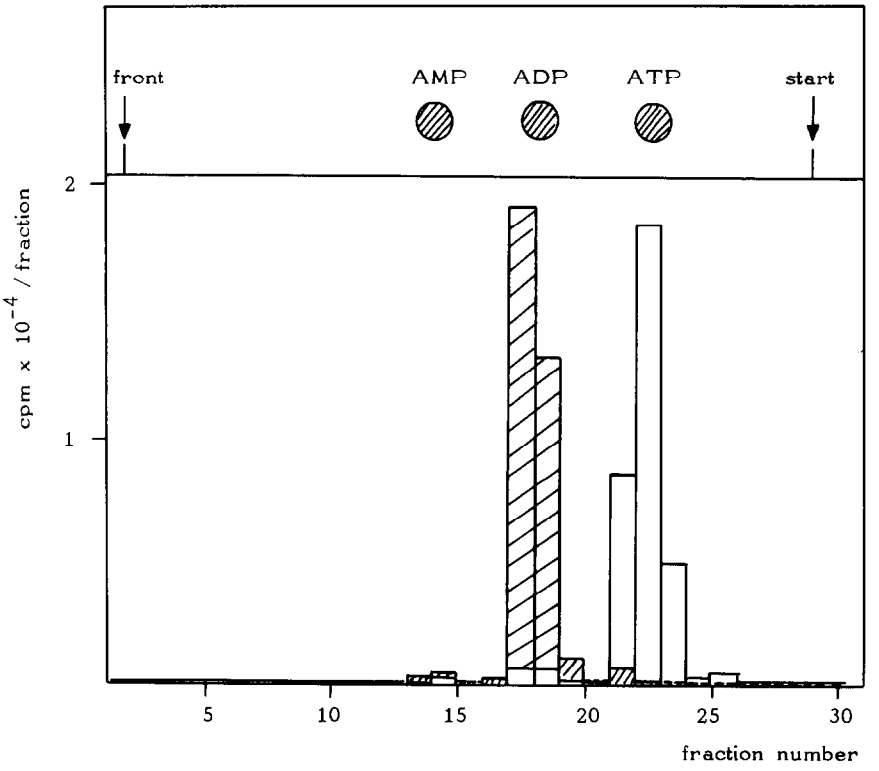

Fig. 3. Enzymic displacement of ATP radioactivity to the ADP position by the hexokinase reaction. 0.4 μCi [^{3}H] ATP (= 47 pmoles) were incubated in the presence of non-labeled HeLa cell extract, containing 5.7 nmoles ATP, with 0.91 μg hexokinase for 30 min at 37°C. 10 μl of the incubation mixture were separated on PEI cellulose chromatography. For experimental details see Materials and methods. (shaded columns = + hexokinase). The upper part indicates the position of the UV markers.

of HeLa suspension cultures with [^{3}H] uridine and [^{3}H] adenosine respectively.

Fig. 5A shows the kinetics of UTP specific radioactivities in HeLa cells during incubation with 1×10^{-5} M or 2×10^{-4} M [^{3}H] uridine. At 1×10^{-5} M exogenous uridine UTP specific radioactivity reached values remaining 40 to 50% below the uridine values (dotted line), whereas at 2×10^{-4} M, UTP nearly reached the uridine specific radioactivity. This different response of UTP specific radioactivity to the two uridine concentrations is due to the contribution of de novo pyrimidine nucleotide synthesis becoming suppressed by exogenous uridine only at concentrations $> 10^{-4}$ M [2]. In contrast to the pyrimidine nucleotide synthesis the endogenous synthesis of ATP is nearly completely suppressed by 1×10^{-5} M concentrations of exogenous adenosine (fig. 5B).

It is interesting to note that in both cases at least 1/3 of the generation time (~ 8 hr) is required to reach steady state values of specific radioactivity of the nucleic acid precursors (cf. [5]). These data clearly show that even short-term incorporation studies of exogenous precursors relying on the assumption of unaltered pool sizes of precursor nucleotides may be subject to serious error of interpretation.

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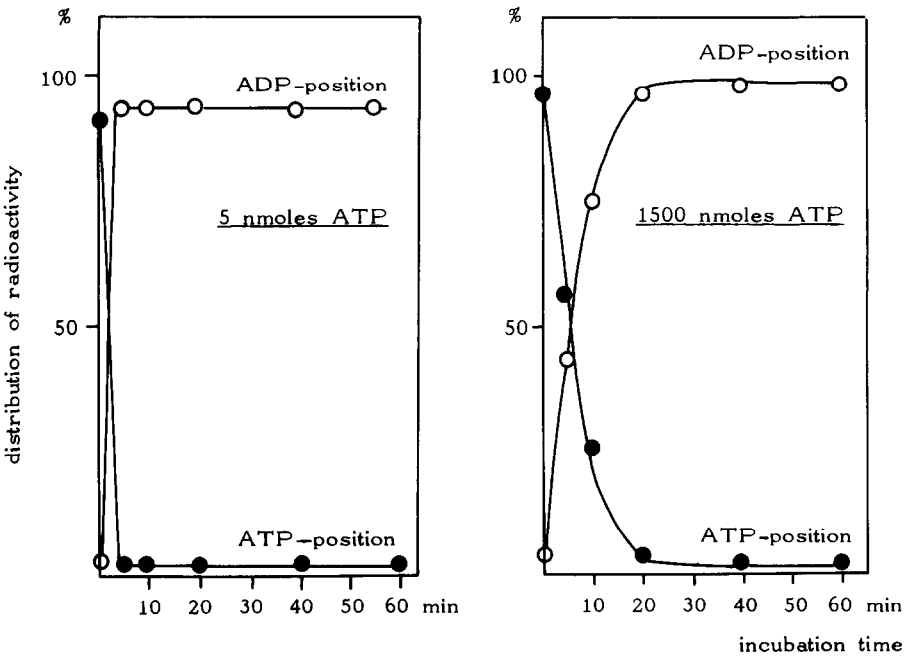

Fig. 4. Kinetic analysis of ATP displacement at two different ATP concentrations. 0.4 μCi [^{3}H] ATP (= 47 pmoles) were incubated in the presence of 5 nmoles (left panel) and 1500 nmoles (right panel) non-labeled ATP at 37°C with 0.91 μg hexokinase. 10 μl of the incubation mixture were separated on PEI cellulose chromatography as described in Materials and methods.

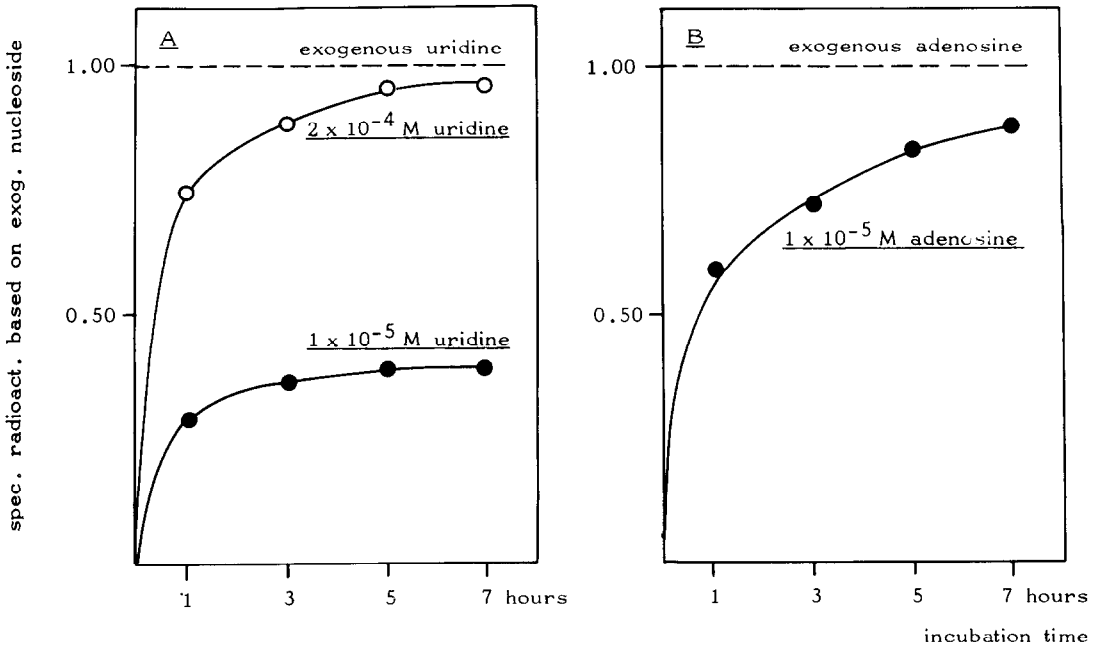

Fig. 5. Kinetics of UTP and ATP specific radioactivities with exogenous [^{3}H]uridine (A) and [^{3}H]adenosine (B), respectively, in HeLa cultures. HeLa suspension cultures (~ 0.3 X 10^6 cells/ml) were incubated with [^{3}H]uridine and [^{3}H]adenosine, respectively, at the given concentrations. At the times indicates 100 ml of the suspension (~ 3 X 10^7 cells) were taken, the acid soluble fraction prepared and analyzed for UTP and ATP specific radioactivities, as described in Materials and methods.

References

[1] Kaukel, E., Fuhrmann, U. and Hilz, H. (1972) Biochem. Biophys. Res. Commun. 48, 1516–1524.
[2] Kramer, G., Wiegers, U. and Hilz, H. (1973) Biochem. Biophys. Res. Commun. 55, 273–281.
[3] Wiegers, U. and Klapproth, K., submitted for publication.
[4] Wiegers, U., Kramer, G., Klapproth, K., Rehpenning, W. and Hilz, H., submitted for publication.
[5] Kramer, G., Klapproth, K. and Hilz, H. (1972) Biochim. Biophys. Acta 262, 410–419.
[6] Schlaeger, C., Hoffmann, D. and Hilz, H. (1969) Hoppe-Seyl. Z. Physiol. Chem. 350, 1017–1022.
[7] Keppler, D., Rüdiger, J. and Decker, K. (1970) Anal. Biochem. 38, 105–114.
[8] Lamprecht, W. and Trautschold, I. (1970) in: Methoden der enzymatischen Analyse, (H. U. Bergmeyer, ed.), Vol. 2., 2024–2033, Vlg. Chemie, Weinheim.
[9] Randerath, K. and Randerath, E. (1967) in: Methods in Enzymology, Vol. 12, part A, eds. (L. Grossmann and K. Moldave, eds.), 323–349 Academic Press, New York and London.
[10] Kuhn, Ch. and Hilz, H. (1973) Hoppe-Seyl. Z. Physiol. Chem. 354, 521–526.
[11] Randerath, K. (1962) Angew. Chemie 74, 484–488.