SIMULTANEOUS DETERMINATION OF THE RATES OF DNA AND PROTEIN SYNTHESIS *IN VIVO* WITH DOUBLE LABELING OF THE BIOPOLYMERS AND THEIR PRECURSORS

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Analysis of coupled processes *in vivo* is a problem of ever-increasing urgency. The task of simultaneous determination of levels of DNA and protein synthesis is made all the more urgent because the balance between the velocities of synthesis of these biopolymers is an important factor in normal physiological activity, for it is an essential condition for DNA repair both in normal and in various pathological states [5]. Combined determination of ^3H and ^{14}C in biological samples presents considerable difficulties [9]. To avoid errors connected with differences in the biological dilution of the precursors [4, 12], when the rate of synthesis of biopolymers is determined the intensity of labeling of the biopolymer must be related to the biological dilution of the corresponding precursor in the labeling period [4]. The writer has developed a method of simultaneous determination of the rates of DNA and protein synthesis which satisfies the above demands.

EXPERIMENTAL METHOD

Experiments were carried out on hybrid (CBA × C57BL)F_1 mice weighing 18-20 g. Each mouse was given a subcutaneous injection of 0.74 MBq of [^3H]thymidine (from "Izotop," USSR) with specific activity of 1.554 TBq/mmole, and 0.074 MBq of [^{14}C]leucine (from "Izotop," USSR) with specific activity of 1.48 MBq.

Radioactivity was determined on a Delta-300 liquid scintillation counter (from Trakor Analitik). Two scintillation mixtures were used: 1) 0.2 g POPOP, 4 g PPO, 100 ml absolute ethyl alcohol, 10 ml ethyleneglycol, and 40 g naphthalene to 900 ml dioxane; 2) 60 g naphthalene, 4 g PPO, 0.4 g POPOP, 20 ml methyl alcohol, and 50 ml Triton X-100 (from Ferak, Berlin) to 900 ml dioxane.

After injection of the isotopes the mice were decapitated and the spleen homogenized in 2 ml of standard citrate buffer [10]; 1 ml of homogenate was used to determine specific activity of deoxynucleosides (DN) and amino acids (AA) — fraction 1. Specific radioactivity of protein was determined in 0.5 ml of homogenate — fraction 2; 0.5 ml of homogenate was used to determine specific activity of DNA — fraction 3.

To isolate low-molecular-weight precursors (fraction 3) 1 ml of homogenate was precipitated by 6 ml of 1% picric acid, after which the supernatant was passed through a column with Dowex 1 × 8 resin (100-80 mesh) in the Cl^--form. The resulting solution was evaporated and dissolved in 1 ml distilled water. The amino acid content was determined by the ninhydrin reaction [13], DN by a microbiological method [7]. To determine the radioactivity of [^3H]-DN and [^{14}C]-AA, 0.3 ml of the resulting solution was added to 6 ml of scintillation mixture.

To obtain the protein preparation (fraction 2) 0.5 ml of spleen was precipitated with an equal volume of 10% TCA, the samples were centrifuged, the residue was treated with 0.5 ml of 5 M KOH, and hydrolysis was conducted for 2.5 h in an incubator at 63°C. After cooling, to neutralize the digest 0.5 ml of 50% $HClO_4$ was added. The residue was separated by centrifugation and the protein content [6] and radioactivity of ^{14}C were determined in the supernatant. To determine the radioactivity of ^{14}C, 0.3 ml of protein digest was added to 6 ml of scintillation mixture 2.

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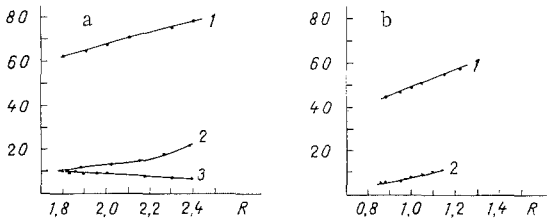

Fig. 1. Curves showing dependence of counting efficiency of radioactivity of ^{3}H and ^{14}C radionuclides on ratio of channels of external standard (R). a: 1) Fraction 1, ^{14}C in carbon channel, K = 0.99, M = 1.7; here and subsequently: K) coefficient of correlation, M) mean relative error of approximation of experimental values of counting efficiency of radioactivity, by theoretical curves, calculated by the equation:

$$M = \frac{1}{N}\left(\sum_{i=1}^{n} \frac{Y\,\text{theor}_i - Y\,\text{exp}_i}{Y_{\text{theor}\,i}} \cdot 100\right);$$

2) fraction 1, ^{3}H in tritium channel, K = 0.93, M = 9.1; 3) fraction 1, ^{14}C in tritium channel, K = 0.94, M = 10.3. b: 1) Fraction 2, ^{14}C in carbon channel, K = 0.97, M = 1.6; 2) fraction 3, ^{3}H in tritium channel, K = 0.97, M = 7.0. Abscissa, ratio of channel of external standard; ordinate, counting efficiency (%).

DNA was isolated by the method in [10]. The isolated DNA was hydrolyzed in 1 ml of 5% $HClO_4$. The DNA content in 0.5 ml of digest was determined by the method in [6]. Radioactivity of ^{3}H was determined in the other half of the digest. For this purpose, 0.5 ml of DNA digest was added to 6 ml of scintillation mixture 1.

EXPERIMENTAL RESULTS

Since [^{3}H]thymidine and [^{14}C]leucine were injected simultaneously into the animals both radionuclides — ^{3}H and ^{14}C — were present in the samples. To determine radioactivity of the isotopes the method of ratio between channels of an external standard [8, 9] was used.

Radioactivity of each series of samples was measured twice, and after the first measurement 3.7 kBq of ^{3}H or ^{14}C was added to the samples. Since the relative radioactivity count of the samples after addition of the radionuclides was 100 times or more greater than the initial radioactivity of the samples, no correction was made for the initial radioactivity of the samples.

The results of the second measurement were analyzed by the method of least squares. The results were used to plot curves characterizing dependence of counting efficiency on the ratio of the channels of the external standard and calculation of the mean relative error of approximation. The theoretical curves thus obtained and corresponding approximation errors are shown in Fig. 1: the maximal mean relative error of approximation of the experimental values for the efficiency of counting of radioactivity by the theoretical curves was 10%, which corresponds to an error of 1% for a counting efficiency of 10%.

To create optimal conditions for measuring radioactivity of the samples two modules were used — standard (^{3}H and ^{14}C) and variable.

To measure radioactivity of [^{14}C]-AA and [^{3}H]-DN simultaneously in fraction 1 the variable module was used: Its channels were so selected that the counting efficiency of ^{14}C radioactivity in the tritium channel did not exceed 10% and the counting efficiency of ^{3}H radioactivity in the carbon channel did not exceed 0.5%. These conditions were satisfied by the 10th to the 130th channels (window A) and the 180th to the 450th (window B) of the variable module.

The maximal relative count in the tritium channel in our samples was 650 cpm, and in the carbon channel 708 cpm. With the counting efficiency of ^{14}C in the carbon channel and with a ratio of 64% of the channels of the external standard (Fig. 1), the radioactivity of ^{14}C

TABLE 1. Specific Radioactivity (in %) of DNA, Protein, and Their Precursors under Normal Conditions and in Hypothermia

Experimental conditions	DN	DNA	Protein	AA
Control	100±17,87 (2,4 Bq/mole)	100±10,28 (5,12 Bq/μg)	100±7,17 (15,65 Bq/mg)	100±13,91 (3,28 Bq/μmole)
Hypothermia	137±22,2	15,84±3,15	17,27±3,81	162,2±23,54

Legend. Mean absolute values of specific radioactivity given in parentheses. Number of animals tested 10-18.

was found: it amounted to 1106 cpm. With the counting efficiency of ^{14}C in the tritium channel of 11% (Fig. 1), the relative ^{14}C count in the tritium channel was 112 cpm. Subtracting the relative count of ^{14}C from the relative count of radioactivity in the tritium channel, the relative count of radioactivity of ^{3}H was obtained. The counting efficiency of ^{3}H for the given sample was 14%, so that the absolute radioactivity of ^{3}H in 0.3 ml of the sample was 3771 cpm. Since the counting efficiency of the ^{3}H radioactivity in the carbon channel did not exceed 0.5%, the maximal relative count of ^{3}H in the carbon channel was not more than 19 cpm. The relative count of ^{14}C in the carbon channel was thus 689 cpm (708 − 19 = 689). The relative count of ^{3}H was 2.8% of the relative count of ^{14}C. Consequently, the contribution of tritium to the relative count in the carbon channel can be disregarded; all the radioactivity recorded in the carbon channel therefore belonged to carbon.

To measure ^{14}C in fraction 2 the variable module was used: its channels were so selected that the counting efficiency of ^{3}H in the carbon channel did not exceed 0.5%. These conditions were satisfied by the 10th to the 110th channels (window A) and the 110th to the 450th channels (window B). The maximal relative count in the tritium channel in our samples was 186 cpm, and during counting in the carbon channel 95 cpm (background 30 cpm). The counting efficiency of ^{14}C in the carbon channel at this given ratio between channels of the external standard was 48% (Fig. 1), hence the radioactivity of ^{14}C was 198 cpm. With a counting efficiency of ^{14}C in the tritium channel of 15%, the relative count of ^{14}C was 30 cpm. Subtracting the relative count of ^{14}C from the relative count in the tritium channel, a relative count of ^{3}H radioactivity of 156 cpm was obtained (186 − 30 = 156). The counting efficiency of ^{3}H for this given sample in the tritium channel was 15% and the absolute ^{3}H radioactivity in 0.3 ml of sample was 1040 cpm. Since the counting efficiency of ^{3}H radioactivity in the carbon channel did not exceed 0.5%, the maximal relative count of ^{3}H in the carbon channel was not more than 5 cpm. The relative count of ^{14}C in the carbon channel was thus 90 cpm (95 − 5 = 90). Hence it follows that the maximal relative ^{3}H count was 5.3% of the ^{14}C count.

Consequently, the contribution of tritium to the count in the carbon channel is negligibly small; all the radioactivity recorded in the carbon channel thus belonged to carbon.

To measure the radioactivity of ^{3}H-labeled DNA in fraction 3 a standard ^{3}H and ^{14}C module was used. The count in the carbon channel was at the background level ($P < 0.05$), and for that reason the whole count of radioactivity in the tritium channel belonged to tritium. The curve of counting efficiency of ^{3}H is shown in Fig. 1 (curve 5).

It follows from the results that the suggested approach enabled the radioactivity of DNA, protein, and their precursors to be determined separately with high accuracy by double labeling of the corresponding compounds.

To verify the validity of this approach for determining the rate of synthesis of biopolymers (this parameter is not identical with the level of incorporation of the label), a comparative study was made of these same parameters in normal animals and in animals in a state of deep hypothermia, when it would be reasonable to expect a different dilution (compared with normal) of the compounds with their endogenous analogs [11]. The results of one series of experiments on mice in a state of deep hypothermia (16-18°C) 35 min after injection of labeled precursors are given in Table 1. Hypothermia was induced by cooling the anesthetized animals [3].

It follows from the data described above that the level of incorporation of precursors into DNA and protein under conditions of hypothermia was 15.8 ± 3.15 and 17.3 ± 3.81% of normal respectively. Hence the velocity of synthesis of DNA and protein in the experimental

and control series (expressed relatively) was found by introducing a correction for dilution of the precursors [4]. The rate of synthesis of DNA and protein was 11.6 and 10.7% of normal respectively. According to our data, inhibition of DNA and protein synthesis thus takes place parallel to one another. These findings differ from data published previously [1], according to which DNA synthesis during hypothermia is inhibited by a much greater degree than protein synthesis (33.6 and 49.1% of normal respectively at 20-21°C and 13.9 and 25.6% at 14-15°C). The probable cause of the differences is that in the present experiments the error introduced by unequal dilution of the precursors at normal temperature and during hypothermia was excluded [2].

The parallel trend in the decrease in velocity of macromolecular synthesis during hypothermia, produced in the course of evolution, is an indirect argument in support of the important role of the factor of balance in the inhibition of macromolecular synthesis in repair processes.

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