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A rapid enzymic procedure for the determination of picomole amounts of UDP-glucuronic acid

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A simple microassay for the determination of UDP-glucuronic acid was developed on the basis of the formation of benzo[*a*]pyrene 3-glucuronide catalysed by UDP-glucuronyltransferase of guinea-pig liver. As little as 1–5 pmol of UDP-glucuronic acid was detectable in extracts of heat-denatured probes of liver or cultured cells equivalent to 10–50 μg of cellular protein.

In studying UDP-glucuronyltransferase activity in cells in monolayer culture we were faced with the problem of quantifying its cofactor UDP-glucuronic acid in severely limited amounts of cellular material. The methods available for the determination of UDP-glucuronic acid (Dutton & Storey, 1962; Dutton, 1966; Bock *et al.*, 1977; Wong, 1977; Lehnert *et al.*, 1979) are not sensitive enough to work with the 0.5–5.0 mg wet wt. of cellular material contained in monolayer cultures under our experimental conditions. We therefore developed, and in the present paper describe, an assay capable of detecting picomole amounts of UDP-glucuronic acid in as little as 50 or 200 μg wet wt. of liver or cultured cells respectively. This was achieved by using UDP-glucuronic acid extracted from fresh tissue as donor substrate for guinea-pig liver UDP-glucuronyltransferase as done by others (Bock *et al.*, 1977; Wong, 1977; Lehnert *et al.*, 1979); however, we employed 3-hydroxybenzo[*a*]pyrene as the acceptor substrate because the resulting glucuronide can be measured down to 5 pmol quantities owing to its high coefficient of fluorescence (Singh & Wiebel, 1979).

Materials and methods

Chemicals

UDP-glucuronic acid (sodium salt; 98–100% pure) was obtained from Sigma Chemical Co. 3-Hydroxybenzo[*a*]pyrene was obtained from the Carcinogenesis Standard Reference Compound Bank, NCI, Bethesda, MD, U.S.A. All other chemicals used were of analytical grade.

Preparation of hepatic microsomal fraction

Hepatic microsomal fraction from male guinea pigs (approx. 400 g body wt.) was used as a source of UDP-glucuronyltransferase because the fraction contained only negligible amounts of pyrophosphatase directed toward UDP-glucuronic acid (Wong & Lau, 1970). The microsomal fraction was prepared by the method of Wiebel *et al.* (1971). The fraction was washed twice by re-suspension in 0.25 M-sucrose, gentle homogenization (Potter–Elvehjem glass/Teflon homogenizer) and centrifugation at 105000 $g_{av.}$ for 60 min (Beckman L-2 65B). At each step portions (0.5 ml) were stored at −70°C. UDP-glucuronyltransferase activity toward 3-hydroxybenzo[*a*]pyrene was stable for at least 1 month when stored at −70°C. Twice-washed microsomal fraction was used throughout the experiments as a source of the enzyme, if not stated otherwise.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Cell cultures

Monolayers of H-4-II-E (hepatoma, rat), RAG (renal adenoma, mouse), BHK21/C13 (kidney, Syrian hamster) and V79 (lung, Chinese hamster) cells were grown in plastic dishes (10 mm × 60 mm). The source of these cell lines and their growth conditions have been described previously (Wiebel & Singh, 1980).

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Preparation of extracts from cells in culture or liver

Cultured cells. Plates taken from the incubator were placed on ice. The growth medium was siphoned off and 1.0ml of cold water (0–4°C) was added. The cells were scraped off and collected into a test tube, which was immediately placed in boiling water. The samples were cooked for 3min to denature the protein (Dutton, 1966), cooled down in ice and centrifuged at approx. 3000*g*. The supernatant served as a source of UDP-glucuronic acid.

Liver. Male C3H mice (approx. 30g body wt.) were used for the preparation of liver extract. After decapitation, a portion of the liver was excised and placed on a balance. On recording the weight, small pieces (approx. 40mg) were excised and dropped individually into 5ml of boiling water. The handling of liver tissue up to the last step took not more than 15–30s. The tissue was cooked for 3min, cooled down and homogenized (Potter–Elvehjem, glass/Teflon homogenizer). After brief centrifugation at approx. 3000*g* portions of the supernatant were diluted with water to a final 5mg wet wt. of tissue/ml. The material was either immediately used or stored at −70°C for later assays.

Enzymic determination of UDP-glucuronic acid

UDP-glucuronic acid-dependent formation of benzo[*a*]pyrene 3-glucuronide was determined as described previously (Singh & Wiebel, 1979). The reaction mixture contained in a volume of 200μl: 20μmol of Tris buffer, pH7.6; 1μmol of $MgCl_2$; 0.01% Brij-58 (w/v); 10nmol of 3-hydroxybenzo[*a*]-pyrene in 10μl of methanol; 50μl of tissue extract as a source of UDP-glucuronic acid and 50μg of guinea-pig liver microsomal protein. The reaction mixtures were assembled in test tubes immersed in ice. The tubes were incubated for 30min in a water bath at 37°C with mild shaking. The reaction mixtures were extracted with 6ml of chloroform/methanol (2:1, v/v) together with 0.8ml of water by vigorous shaking for 5–10s. Fluorescence of the product, benzo[*a*]pyrene 3-glucuronide, in the aqueous-methanol phase was measured at 378nm excitation/425nm emission with a Jobin Yvon JY 3D spectrofluorimeter. The amount of UDP-glucuronic acid in the tissue extract was estimated as described below.

Results and discussion

UDP-glucuronic acid-dependent formation of benzo[*a*]pyrene 3-glucuronide reached its maximum in less than 15min of incubation in the presence of 50–500pmol of the cofactor and of 50μg of guinea-pig liver microsomal protein (Fig. 1). At incubation times longer than 30min, fluorescence intensity due to the glucuronide showed a slight decline. The relative fluorescence was proportional

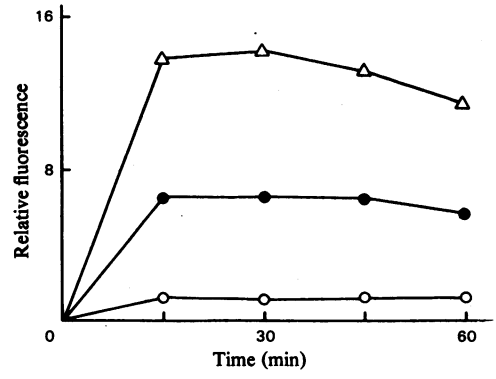

Fig. 1. *Time dependency of benzo[a]pyrene 3-glucuronide formation with various amounts of UDP-glucuronic acid*
Portions of UDP-glucuronic acid (○, 50pmol; ●, 250pmol; △, 500pmol) were incubated with 50μg of microsomal protein for various time periods. Other conditions were as described in the Materials and methods section. Points represent the means of three observations. The coefficient of variation was less than 5%.

to the amounts of UDP-glucuronic acid between 5 and 1000pmol (Fig. 2). UDP-glucuronic acid in tissue probes can be quantified by extrapolation from standard curves obtained with known amounts of UDP-glucuronic acid. In view of the linearity of the reaction in a wide range of UDP-glucuronic acid concentrations (Fig. 2) one or two standard values should suffice. The reaction has been shown to be specific for UDP-glucuronic acid and not to be influenced by other UDP derivatives (Wong, 1977; Lehnert *et al.*, 1979). The recovery of UDP-glucuronic acid from tissue extracts was measured by addition of various amounts of the cofactor to monolayer cells shortly before their collection and heat denaturation. Comparison with the 'external' standard indicates that under these conditions about 80% of the UDP-glucuronic acid is recovered at all concentrations tested (Fig. 2).

The sensitivity of the present assay is determined by the amount of UDP-glucuronic acid that is carried into the reaction mixture by the guinea-pig microsomal fraction. Extracts prepared from unwashed microsomal fraction were shown to contain 500–650pmol of UDP-glucuronic acid/mg of protein. Washing of the microsomal fraction decreased contaminating UDP-glucuronic acid to a value of 130–140pmol/mg of protein, which could not be significantly decreased by a second washing. Thus, under standard conditions, i.e. with 50μg of microsomal protein, the background amounts to 5–7pmol of UDP-glucuronic acid.

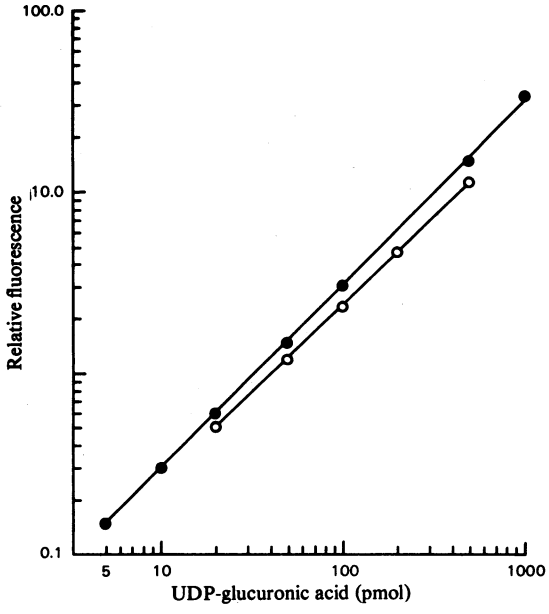

Fig. 2. *Dependency of relative fluorescence intensity on UDP-glucuronic acid concentration*

A standard curve (●) was established by incubating 50μg of microsomal protein with various amounts of UDP-glucuronic acid. To determine the recovery of UDP-glucuronic acid (○) from extracts of H-4-II-E cells the procedures described in the Materials and methods section were followed except that known amounts of UDP-glucuronic acid were added to the water used for collecting the cells. The results give the fluorescence intensity minus the fluoresence arising due to the presence of cellular UDP-glucuronic acid, i.e. product formation in the absence of exogenous UDP-glucuronic acid (see Table 1). Points represent the means of duplicates from each of two culture plates. The coefficient of variation was less than 5%.

Table 1. *UDP-glucuronic acid content of mouse liver and cells in culture*

Preparation of tissue extracts and determination of UDP-glucuronic acid are described in the Materials and methods section. Samples were assayed in duplicate. The coefficient of variation was in general less than 5%.

Tissue	UDP-glucuronic acid (nmol/mg of protein)
C3H mice	
A	1.80 ± 0.20*
B	2.85 ± 0.20
C	2.53 ± 0.30
D	2.18 ± 0.02
E	3.49 ± 0.16
Cell lines	
H-4-II-E	2.05 ± 0.20†
RAG	0.79 ± 0.10
BHK21/C13	0.34 ± 0.03
V-79	0.37 ± 0.05

* Means and range of determinations from two tissue probes from each liver.

† Mean and range of six observations from two separate experiments of three plates each.

The applicability and reproducibility of the assay were tested in extracts from cultured cells and from liver (Table 1). Measurements on mouse liver were made with extracts from 50μg wet wt., equivalent to 8–10μg of protein. UDP-glucuronic acid concentrations in five livers ranged from 1.8 to 3.5 nmol/mg of protein; however, they did not differ significantly in duplicate probes from individual animals. Likewise, triplicate cultures of four cell lines varied less than 15% in their UDP-glucuronic acid concentrations although the cell lines differed at least by a factor of 5 in their UDP-glucuronic acid content (Table 1). The extracts of cells used in these determinations corresponded to 50–100μg of protein, which is equivalent to about one-tenth of a semiconfluent monolayer of cells from a small culture dish (60mm diameter). The coefficient of variation of the assay using a given tissue extract or extraneous UDP-glucuronic acid was generally less than 5%.

The use of 3-hydroxybenzo[*a*]pyrene in the present assay improves the sensitivity of the previously available procedures (Dutton & Storey, 1962; Wong, 1977; Bock *et al.*, 1977; Lehnert *et al.*, 1979) by one or two orders of magnitude. The high coefficient of fluorescence of the reaction product, benzo[*a*]pyrene 3-glucuronide, allows measurements to be taken in the picomole range. This in turn offers the advantage of employing low amounts of microsomal fraction, minimizing the carry-over of residual UDP-glucuronic acid and, hence, the background value. Probes of tissues of <1 mg wet wt., which suffice for the assay, are readily denatured and extracted. Preparation of samples and determination of the benzo[*a*]pyrene 3-glucuronide involve only two or three straightforward extraction and dilution steps. The entire procedure may be completed within a few hours if samples are not stored at various time points for later processing.

Sensitivity and simplicity recommend the present assay for routine determination of UDP-glucuronic acid in large numbers of samples as well as in tissue probes that are available only in minute quantities such as biopsies.

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