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METABOLISM OF DRUGS IN ISOLATED RAT HEPATOCYTES*

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Abstract—Enzymatic activities for the *N*-demethylation of aminopyrine, the *p*-hydroxylation of aniline and the UDP-glucuronidation of *p*-nitrophenol in isolated rat hepatic cells (intact and disrupted by ultrasonication) have been compared on the basis of DNA content to those in homogenates from fresh liver tissue and the crude microsomal fractions prepared from these homogenates. The influence of different preparative procedures on the enzymatic activities are discussed. The inhibitor of drug metabolism SKF 525 A blocks the UDP-glucuronidation only in the intact isolated cells and not in the homogenates. In contrast, *N*-demethylation is inhibited by SKF 525 A both in the intact cells and in the disrupted cells and in 9000 *g* supernatants from fresh liver homogenates. This indicates that for the *N*-demethylation a true enzyme inhibition is involved while for the UDP-glucuronidation a non-specific change in the properties of the membranes leading to a reduced permeability for substrate or cosubstrate is more probable. The suspension of isolated hepatocytes appeared to be suitable to study various aspects of drug metabolism, especially their modifications due to changes in the cellular integrity and changes in the permeability of the membranes.

IN RECENT years numerous investigations have been performed on biotransformation of drugs, with on the one side the whole animal as the subject and on the other the liver homogenate or microsomal cell fraction. Experiments on total animals and more recently on perfused livers have provided us with considerable information concerning the metabolic fate of many drugs, but, because of the complexity of the whole organism, it will be difficult to evaluate the experimental results with respect to the enzymatic reactions involved.[1–4] Although a number of enzymatic mechanisms have been characterized by *in vitro* experiments carried out with more or less purified enzyme systems and subcellular fractions, these results cannot be correlated directly to the *in vivo* situation. In this respect a study of drug metabolism on the level of the intact but isolated hepatic cells may fill up the gap. Some attention has been paid to the study of drug metabolism in liver slices.[5–7] We, however, prefer isolated liver cells to liver slices, since—it depends on the thickness of the slices—either the free diffusion of substrates and metabolites may be limiting and, moreover, a large fraction of the cells of the slices has been ruptured. The study described in this paper has to be regarded as an attempt to design a system of isolated liver cells, not subject to variations imposed *in vivo* by nerve, blood or hormonal supply, in which drug metabolizing capacities and the modification thereof can be investigated on the cellular level. The

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N-demethylation of aminopyrine, the hydroxylation of aniline and the glucuronidation of *p*-nitrophenol have been compared in liver homogenates, crude microsomal fractions prepared in different ways and in isolated liver cells.

EXPERIMENTAL PROCEDURE

Animals

Male rats (Wistar) weighing about 180–220 g were used.

Isolation of the hepatocytes

Heparinized animals were given ether anesthesia and the livers were perfused *in situ* with about 60 ml of an ice-cold saline solution. The perfused livers were rapidly excised, washed with the perfusion fluid and sliced into cubes of about 2 mm^3. After washing twice in succession with a calcium-free Tyrode solution the small liver pieces were incubated at 37° for 1 hr in a dissociating medium following Segard *et al.*[8] consisting of inactivated bovine serum containing sodium citrate (3×10^{-2}M), sodium adenosine-5′-triphosphate (10^{-3}M) and $MnCl_2$(10^{-3}M). During the dissociation process the liver suspension was agitated by magnetic stirring. After incubation the suspension was filtered once through a Kleenex tissue to remove strands of connective tissue and non-dissociated pieces of liver tissue. At this stage the suspension contained apart from the separated hepatocytes a number of cell fragments and erythrocytes. The intact hepatocytes were separated by differential centrifugation at low speeds. The final cell sediment was resuspended in a known volume of ice-cold calcium-free Tyrode solution (Fig. 1). Cell counts were made on a hemocytometer (Bürker–Türk). The yield varied from $10–30 \times 10^6$ cells per gram liver. Enzyme activities were tested both in intact cells and cell homogenates. Homogenates from isolated cells were prepared by treatment of the suspended hepatocytes in an ultrasonic desintegrator (MSE-100 Watt).

Preparation of liver homogenates and crude microsomal fractions

As far as enzymatic activities in fresh livers are concerned, these were measured in crude microsomal fractions prepared from liver homogenates. Rats were killed by decapitation, the livers were rapidly excised and 20% liver homogenates were prepared at 1–4° with a Potter–Elvehjem homogenizer in 0·25 M isotonic sucrose containing 10^{-3} M sodium ethylenediamine tetraacetate (EDTA-Na) and 5×10^{-2} M Tris (hydroxymethyl) aminomethane HCl buffer pH 7·4.

The homogenates were centrifuged at 9000 *g* for 20 min at 2° and the supernatants were tested for enzymatic activities.

Respiration measurements

Oxygen uptake by liver homogenates and isolated cells was measured manometrically in a Warburg respirometer. The homogenates or the cells suspended in Tyrode solution (pH 7·4) were incubated for 30 min at 37° with air as the gas phase in the presence of ATP (10^{-3} M) and sodium succinate (10^{-2} M).

Enzymatic assays

N-*demethylation of aminopyrine.* Optimal conditions for testing *N*-demethylating activities in 9000 *g* supernatants from liver homogenates have been described earlier by Dewaide and Henderson.[9] The assay media contained 5×10^{-2} M Tris-HCl

(pH 8·0), 5×10^{-3} M semicarbazide, 8×10^{-4} M $MgCl_2$, 8×10^{-6} M $MnCl_2$, 5×10^{-3} M sodium isocitrate, 10 mg isocitric dehydrogenase per ml (Sigma type 4, capable of reducing $5{\cdot}7 \times 10^{-3}$ m-moles NADP per minute per mg) and $8{\cdot}8 \times 10^{-5}$ M NADP. In order to ensure reduction of all NADP to NADPH the assay media were preincubated for 10 min.

Aminopyrine ($16{\cdot}7 \times 10^{-3}$ M) was used as a substrate. The reaction was started by adding the 9000 *g* supernatant to this assay medium. The total volume of the reaction mixture was 3 ml.

In the enzyme assays carried out with isolated cells the reaction was started by adding $3–5 \times 10^6$ cells suspended in a calcium-free Tyrode solution to the assay medium described before. In this incubation mixture thus obtained the isotonicity was provided by increasing the Tris-HCl buffer concentration to 6×10^{-2} M, in order to ensure that the cells stayed intact during the incubation. This was controlled microscopically at the end of the incubation period.

After incubation at 37° the reaction was stopped by the addition of 0·5 ml 25% $ZnSO_4 \times 7H_2O$ and 0·5 ml of a saturated $Ba(OH)_2$ solution.

After protein precipitation the amount of formaldehyde generated in the supernatant was determined according to the method of Nash,[10] as modified by Cochin and Axelrod.[11]

Hydroxylation of aniline. The incubation mixture for the assay of hydroxylating activities had the same composition as the medium mentioned above in the *N*-demethylation test, except for the substrate and coenzyme concentrations, which were for aniline 24×10^{-3} M and for NADP 13×10^{-5} M respectively. Semicarbazide was omitted.

After incubation at 37° the reaction was stopped by the addition of 2 ml ethyl acetate. *p*-Aminophenol was extracted by ethyl acetate and quantitatively determined following to the phenol indophenol method of Brodie and Axelrod.[12]

Glucuronidation of p-*nitrophenol.* The glucuronidation was carried out with slight modifications following the method of Isselbacher.[13] Homogenates and intact cells were incubated at 37° in a mixture containing Tris-HCl buffer (pH 7·4) (5×10^{-2} M), $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M), uridine-5′-diphosphoglucuronate (UDPGA) (3×10^{-3} M), *p*-nitrophenol (7×10^{-4} M) and saccharo-1, 4-lactone (10^{-3} M). The total volume was 1·5 ml. The reaction was stopped by adding 1 ml ethanol. The glucuronide formation was measured indirectly by determining the disappearance of *p*-nitrophenol. For this purpose aliquots of the deproteinized medium were diluted with 0·1 N NaOH and assayed spectrophotometrically at 398 mμ.

DNA assay. DNA was determined by the diphenylamine test following Burton[14] (1956) after a pre-extraction according to Wanka[15] (1962). Calf thymus DNA (BDH) was employed as a reference standard in the calculation of the DNA content. The enzyme activities were expressed per μg DNA.

For all enzyme reactions standard solutions and blanks were carried through the whole procedure in order to get a good quantification of the enzymatic activities.

RESULTS

Viability of the isolated hepatocytes

In order to ensure that the isolated liver cells suspended in calcium-free Tyrode solution are still viable, the rate of oxygen uptake was measured for the cells disrupted

by sonication, for the intact cells and for fresh liver homogenates. The respiratory capacities of the various preparations could be compared per μg DNA (Table 1). It can be concluded that the respiratory capacity is maintained in our isolation procedure.

N-*demethylation of aminopyrine.* Figure 2a illustrates the *N*-demethylation activities of intact and sonicated cells as a function of the time. The enzymatic activity is found to be unchanged during the first 10–20 min of the incubation period and then decreases. The same pattern has been described for the *N*-demethylation activity of the 9000 *g* supernatant from fresh liver homogenates (Dewaide and Henderson[9]).

Table 2 represents the rates of *N*-demethylation by isolated hepatocytes (before and after sonication), total liver homogenates and crude microsomal fractions, measured after 10 min incubation.

TABLE 1. RESPIRATION MEASUREMENTS

	Mean oxygen uptake* per: gram liver	10^6 cells	μg DNA	*n*
Homogenates from fresh liver	108	—	0·052	7
Intact isolated cells	—	0·68	0·052	4
Isolated cells after sonication	—	0·76	0·058	4

* average value expressed as μl O_2 consumed per min.
n number of experiments.

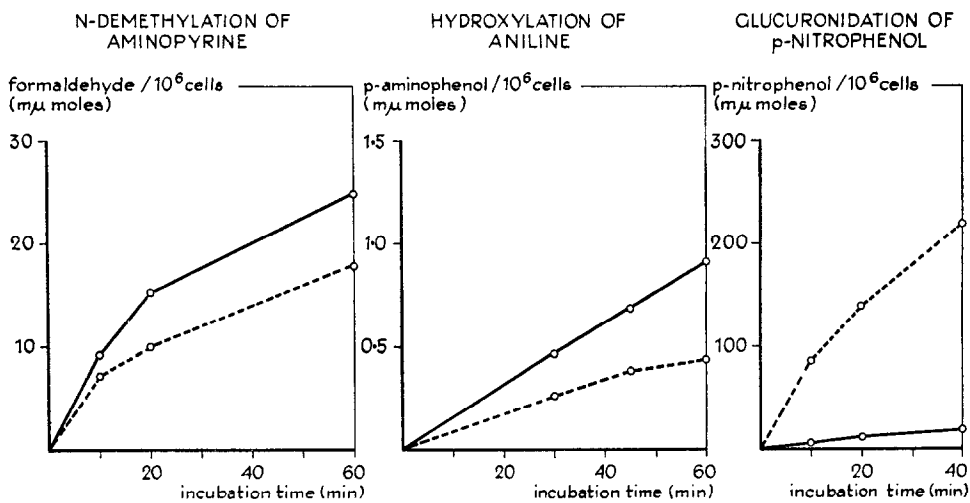

FIG. 2.

(a) The *N*-demethylation of aminoprine by isolated rat hepatocytes. The enzyme activities are expressed as mμmoles formaldehyde produced per 10^6 cells during different incubation periods.
(b) The hydroxylation of aniline by isolated hepatocytes. The enzyme activities are expressed as mμmoles *p*-aminophenol formed per 10^6 cells during different incubation periods.
(c) The glucuronidation of *p*-nitrophenol. Enzyme activity is expressed as mμmoles *p*-nitrophenol conjugated per 10^6 cells during different incubation periods.

○———○ intact cells; ○ - - - ○ sonicated cells.

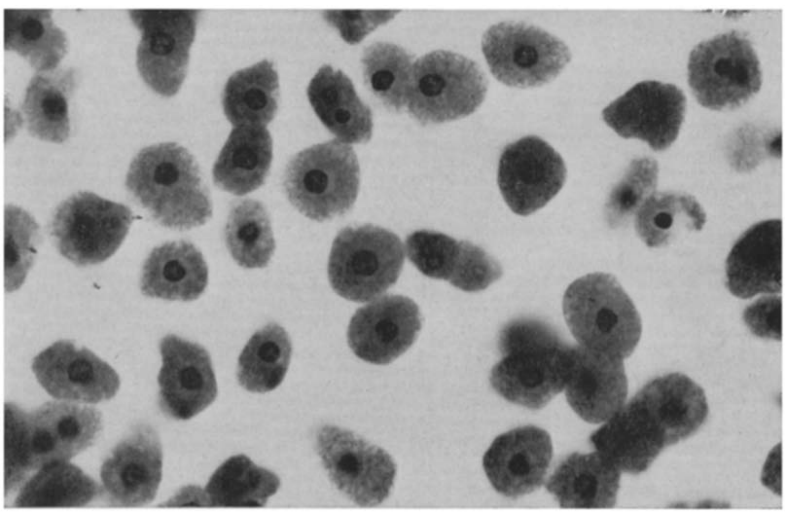

FIG. 1. Photomicrograph of the isolated rat hepatocytes in suspension. (Hematoxylin and eosin, × 360)

TABLE 2. COMPARISON OF SOME DRUG-METABOLIZING ACTIVITIES IN DIFFERENT *IN VITRO* SYSTEMS FROM RAT LIVER

Source of enzyme activity:	*N*-Demethylation of aminopyrine mean activity* per:				Hydroxylation of aniline mean activity† per:				Glucuronidation of *p*-nitrophenol mean activity‡ per:			
	gram liver	10^6 cells	mg DNA	*n*	gram liver	10^6 cells	μg DNA	*n*	gram liver	10^6 cells	mg DNA	*n*
Total homogenates from fresh liver	7·02±1·31	—	3·39±0·80	6	937± 78	—	0·45±0·07	4	34·9±7·4	—	16·9±4·3	9
Crude microsomal fractions§	15·00±2·21	—	7·25±1·49	22	1280±304	—	0·62±0·20	14	26·7±6·5	—	12·9±3·6	6
Intact isolated cells	—	0·058±0·008	4·36±0·98	9	—	1·50±0·80	0·11±0·05	8	—	0·039±0·012	2·9±1·1	8
Isolated cells after ultrasonication	—	0·044±0·008	3·32±0·85	5	—	0·74±0·21	0·06±0·02	4	—	0·393±0·069	29·6±7·5	7

* Expressed as μmoles formaldehyde per hour

† Expressed as mμmoles *p*-aminophenol produced per hour

‡ Expressed as μmoles *p*-nitrophenol conjugated per hour. The UDP-glucuronyltransferase activities in the total homogenates and the crude microsomal fractions were assayed after ultrasonic treatment in contrast with the measurements of the *N*-demethylation and the hydroxylation, which were carried out with extracts prepared without ultrasonication.

n The number of experiments

§ 9000 *g* Supernatants prepared from fresh liver homogenates. For these fractions calculations of the activities per DNA have been based on the amount of DNA determined in the total homogenates before centrifugation at 9000 *g*.

Hydroxylation of aniline. From literature it is known that the enzyme system involved in the hydroxylation of aniline is more stable than the *N*-demethylating system (Schenkman *et al.*[16]). In crude microsomal fractions the hydroxylating activity is stable for 1 hr. In the intact isolated hepatocytes, too, the activity is stable at least for 1 hr; after disruption of the cells the activity declined after 45 min (Fig. 2b). In Table 2 the rates of hydroxylation of aniline and *N*-demethylation of aminopyrine are summarized for various preparations. There is an appreciable loss in activity for the hydroxylation of aniline in the isolated liver cells.

Glucuronidation of p-*nitrophenol.* The amounts of *p*-nitrophenol conjugated by intact isolated cells and disrupted isolated cells for different incubation times are presented in Fig. 2c. There is a striking difference between the activities of intact and sonicated cells. This might be an indication that there is a permeability barrier for the substrate or the cosubstrate, which can be abolished by ultrasonic treatment. The same holds true for fresh liver homogenates and crude microsomal fractions. The mean UDP-glucuronyltransferase activities, expressed as mμmoles *p*-nitrophenol conjugated per hour per μg DNA, were found to be 4·7 $\pm$ 1·6 and 3·8 $\pm$ 1·1 respectively. After sonication these activities increased to 16·9 $\pm$ 4·3 and 12·9 $\pm$ 3·6 mμmoles per hour per μg DNA, the values represented in Table 2. Remarkably the reverse situation holds for the hydroxylation and *N*-demethylation reactions, where the activities of the various preparations decreased about 30 per cent after ultrasonication.

The influence of SKF 525 *A.* β-Diethylaminoethyl diphenylpropylacetate (SKF 525 A) prolongs the activity of a number of drugs because of an inhibition of drug metabolism *in vivo*.[17, 18] For a number of drugs also a block of the *in vitro* transformation by homogenates and microsomal fractions has been described.[1] In the present study the influence of SKF 525 A (10^{-4} M) on the *N*-demethylating and glucuronidating

TABLE 3. INHIBITION BY SKF 525 A

	N-demethylation of aminopyrine (per cent)	Glucuronidation of *p*-nitrophenol (per cent)
Total liver homogenates	—	1 (3)*
Crude microsomal fractions	36 (3)	—
Intact isolated hepatocytes	51 (3)	40 (4)
Disrupted isolated hepatocytes	40 (2)	5 (4)

*The number of experiments is given in parentheses.

capacities of the isolated hepatocytes has been investigated. SKF 525 A has been added to the isolated cells, the homogenates and the 9000 *g* supernatants just before the measurements of the enzymatic activities. This implies that in these experiments only the inhibitory action of the diphasic effect of SKF 525 A can be manifested.[27] The results are summarized in Table 3, where the activity is expressed in per cent of the original activity.

In those cases where the inhibition is the same in the various preparations, true enzyme inhibition is feasible. Large differences in the inhibition for the various preparations possibly indicate an interference of SKF 525 A with the transport of substrate or cosubstrate presumably by changes in the properties of the membranes

involved. The results obtained indicate that the inhibition of the *N*-demethylation presumably is a true enzyme inhibition. The inhibition of the glucuronidation of *p*-nitrophenol for the intact isolated cells is strong as compared to the inhibition for the homogenates. This might be based on an effect of SKF 525 A at the level of the cell membrane such that the permeability for substrate or cosubstrate is reduced.

Changes in the properties of the cell membrane under the influence of SKF 525 A could also be demonstrated by measuring the swelling of the isolated hepatocytes in a hypotonic medium, which is inhibited by SKF 525 A in a concentration of 10^{-4} M. The mean diameter of the isolated hepatocytes in an isotonic solution (0·9% NaCl) is 25·5 $\pm$ 1·0 μ. After staying for 20 min in a hypotonic solution (0·3% NaCl) the mean diameter of these cells increases to 28·6 $\pm$ 0·4 μ; in the presence of SKF 525 A the mean diameter remained 25·5 $\pm$ 0·6 μ. This indicates that SKF 525 A causes a higher osmotic resistance.

DISCUSSION

The method described here for preparing a suspension of isolated hepatocytes yielded recognizable parenchymal cells, when the suspension was examined by light microscopy (Fig. 1). No damage to the morphological integrity of the isolated cells could be observed. Submicroscopical damage to the cell membrane cannot be excluded as a matter of fact.

Comparison of the respiratory capacities of the isolated cells, intact or disrupted by sonication, with that of homogenized fresh liver tissue did not show essential differences.

Comparing the drug-metabolizing activities on basis of DNA content it may be concluded that for the isolated cells the *N*-demethylating activity is about 100 per cent, the hydroxylating activity only about 25 per cent, and the glucuronidating activity about 50 per cent of that of the total homogenate. Ultrasonic disintegration of the total liver homogenate and crude microsomal fraction results in an about 30 per cent decrease of the *N*-demethylating and hydroxylating activities, but in a remarkable, about 4-fold, increase in the glucuronidating activity. The same holds for ultrasonication of the isolated liver cells, where the glucuronidating activity even increases about 10-fold. The remarkable influence of ultrasonication on glucuronidation of *p*-nitrophenol might be explained by the supposition, that beside the outer cell membrane also intracellular elements behave as substrate or cosubstrate barriers, the disorganization of which, for instance by ultrasonication, results in a stimulation of the activity. An increase of the microsomal UDP-glucuronyltransferase activity by treatment with digitonin has been demonstrated recently by Heirwegh *et al.*[19] and by Hänninen.[20]

No conclusive explanation can be given for the difference in the activities of UDP-glucuronyltransferase in the sonicated cells and in the sonicated total homogenates prepared from fresh liver (Table 2).

A number of divergent suggestions can be found in literature to explain the inhibitory action of SKF 525 A on the metabolism of various drugs.[21–23] Our results also suggest that SKF 525 A can act in various ways. The inhibition of the *N*-demethylation of aminopyrine being of the same order of magnitude for the various preparations presumably can be attributed to a true enzyme inhibition as observed previously.[23, 24] The blocking action of SKF 525 A on the glucuronidation of *p*-nitrophenol only takes place in the intact isolated cells (Table 3). The most likely explanation,

therefore, is a change on the level of the cell membrane resulting in, for instance, a reduced permeability for the substrate or cosubstrate. This may also explain the seemingly contrary findings of Cooper *et al.*,[6] who reported an inhibition of the glucuronidation by SKF 525 A in rat liver slices, and of Goldstein *et al.*,[25] who did not find any inhibitory effect of SKF 525 A on the *o*-aminophenol conjugation by liver homogenates. An influence on the cell membrane also becomes apparent from the experiments in which the osmotic resistance of the isolated hepatocytes is found to be increased by SKF 525 A. Similar results were recently reported for human erythrocytes by Lee *et al.*[26] (1968).

The liver parenchymal cell preparation appeared to be suitable for the study of the activity of some of the enzymes representative for the hepatic biotransformation of drugs. Further, modifications of this biotransformation due to certain changes in the cellular integrity and to drugs presumably acting on the permeability of the membranes could be analysed. It might be concluded that a suspension of isolated hepatic cells as described in this paper can be considered as a suitable tool for the analysis of various aspects of drug metabolism at the cellular level.

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