Development of in vitro toxicity tests with cultures of freshly isolated rat hepatocytes

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Summary. Freshly isolated and cultured hepatocytes were analyzed by two-parameter flow cytometry. The combined analysis of DNA and cellular protein content allowed the contribution of ploidy classes and of subpopulations within a ploidy class to be defined. Analysis of hepatocytes during exposure to dimethylsulfoxide (DMSO), phenobarbital (PB), low oxygen tension (4% O_2) or fetal calf serum (FCS), provided insight into the dynamic response of individual ploidy classes as a function of culture time. By analogy with the age-dependent ploidy shifts in vivo, hepatocyte-cultures shift towards adult animals during exposure to DMSO and towards young animals when cultured at low pO_2 (4% O_2). FCS and phenobarbital disturb this constitutive ploidy balance. FCS increased the 2 N cell population, where stem cells probably respond to the proliferative stimuli provided by growth factors in the serum. Phenobarbital affects the liver-specific 4 N hepatocytes, which agrees with effects seen in liver after exposure in vivo. It is suggested that drug-induced pathological alterations in ploidy in hepatocyte cultures could serve as indicators of compounds, such as liver tumor promoters, which interfere with cell differentiation in liver.

The heterotypic cell-cell interaction of freshly isolated hepatocytes with isolated, in vitro cultured, rat liver epithelial cells in co-cultures proved to be a valuable concept in toxicity testing: aldrin epoxidase, an enzyme system involved in xenobiotic metabolism, was stabilized for more than two weeks. After exposure to the three chemicals, 2-acetylaminofluoren, procarbazine and cyproterone-acetate, a preferential toxicity for each compound and cell population was established. Thus heterotypic cell cultures can considerably increase the amount of information available from in vitro studies.

The final concept, combining monitoring of cellular DNA (ploidy) and protein content in hepatocyte cultures during and after exposure to a given test compound at tissue oxygen tension with the heterotypic cell-cell interaction, would create a more in vivo-like culture system. This would enhance the predictability of hepatocyte cultures and contribute to a more widespread use of the test system and as a result help to reduce the number of whole-animal tests.

Key words. Cellular DNA/protein analysis; tissue-like oxygen tension; heterotypic cell-cell interaction.

Introduction

In man the liver plays a key role in drug and xenobiotic metabolism. After absorption from the gut, chemicals are first transported to the liver; there they may be converted to pharmacologically active or toxic metabolites or to hydrophilic excretable conjugates by a broad spectrum of metabolizing enzymes. Therefore, the probability of detecting and characterizing the toxicity of unknown chemicals in vitro is highest in hepatocyte cultures.

Although hepatocytes from humans would be more stable in culture than rodent hepatocytes, rat hepatocytes are used for toxicity testing. This because of the ease in obtaining a sufficient number of cells and because of difficulties associated with obtaining samples from healthy persons and the heterogeneity between individuals. The fact that a number of chemicals which exhibit no mutagenic activity in short-term mutagenicity tests induce hepatic tumors in rodents [1] adds an additional interest to rodent liver cell cultures. They can be used not only to detect hepatotoxins but, in addition, to develop test systems which recognize distinct non-genotoxic steps in carcinogenesis such as disturbances in growth and differentiation [26].

Within the framework of the National Programme for the Replacement of Animals, investigations were focused on threee areas: a) the analysis of hepatocytes at the cellular level by flow cytometry, b) the maintenance of hepatocyte cultures at tissue-like oxygen tension, and c) the maintenance of the xenobiotic metabolism by heterotypic cell-cell interactions.

Hepatocytes can be cultured in plastic dishes, on matrix-coated or uncoated surfaces, on beads, in suspension or on floating membranes and meshes. Monolayer cultures were used for this study. They have the advantage that the morphology of individual cells can be easily observed under the microscope and that direct contact between the test compound and the target is assured and defined.

The goal was to analyze hepatocytes individually, both during an extended culture period and after subtoxic or chronic exposure. Analogous to animal toxicity tests, the reaction of the cells to the toxic insult, rather than the initial acute cell death was studied. Therefore, DNA and protein content of freshly isolated or cultured hepatocytes were simultaneously determined by two-parameter flow cytometry. These measurements include the analysis of the cytoplasm to nuclear ratio [19]. In this paper emphasis is laid on the feasibility of using ploidy shifts as indicators of agents with a potential to interfere with cell differentiation.

Furthermore, an attempt was made to keep culture conditions as close to those existing in vivo as possible. A variable often ignored is the partial pressure of oxygen. Within the lobules in liver a functional and metabolic heterogeneity (metabolic zonation) is present [15, 23]: from

the periportal to the perivenous zone, gradients exist in carbohydrate metabolism[5], xenobiotic metabolism[24] and oxygen tension. In the periportal zone, pO_2 is about 65 mm Hg (9–13% O_2) and reaches 30–40 mm Hg (4–5% O_2) in the perivenous zone. Compound-specific damage within distinct areas of the lobules[31] and within ploidy classes of hepatocytes[47] has been observed. This is believed to be a consequence of the adaption of hepatocyte-metabolism to the micro-environmental conditions present in a particular area of the liver lobules. Therefore, to obtain the most reliable assessment of in vitro hepatoxicity of chemicals, hepatocytes should be exposed under perivenous and periportal conditions. This idea was further stressed by studies which showed that in vitro, tissue oxygen tension affects the genotoxic activity of chemicals[29]. Therefore, periportal (4% O_2) and perivenous (13% O_2) oxygen tensions were established and maintained in various hepatocyte cultures[18], and their influence on ploidy and cellular protein content in hepatocytes was studied.

A major limitation of rodent hepatocyte cultures is their preferential rapid loss of phase I reactions (oxydation, hydroxylation, reduction), particularly those catalyzed by cytochrome P-450-dependent mono-oxygenases. These enzymes are responsible for a variety of important pathways which activate xenobiotics including environmental agents. Numerous attempts have been made to stabilize these enzymes; neither modification of the culture medium (hormones, growth factors, trace elements), exposure to soluble factors (e.g. from mesenchymal cells in conditioned medium), nor variation of the extracellular matrix have met with conspicuous success. Addition of dimethylsulfoxide[20] or of phenobarbital[32] was reported to maintain some of the enzymes involved in xenobiotic metabolism. However, for toxicity testing of unknown chemicals these additives should be replaced, whenever possible, by a system simulating more closely the in vivo conditions, because of unpredictable synergistic effects and undefined side effects on other cellular enzyme activities by these additives.

Co-culture of hepatocytes with epithelial helper cells isolated from 10-day-old rats has proved to be the most promising approach so far[13,14]. In co-culture with freshly isolated hepatocytes, these cells stabilized the metabolic competence of hepatocytes for periods of up to 10 days[4]. Regulation occurred at the transcriptional level[12], and the helper function was organ- but not species-specific. The factor(s) involved are as yet unknown. This concept should be introduced into toxicity testing for measurements of the toxicity of xenobiotics in long-term cultures. Therefore, rat liver epithelial cells (RLEC) were isolated and proliferated in vitro. In co-cultures with hepatocytes, their influence on cellular protein content, intracellular lactate dehydrogenase (LDH) activity and aldrin epoxidase activity was determined[3].

Results obtained more recently are discussed in the context of the already published data.

Materials and methods

Isolation and culture of rat hepatocytes have already been described in detail[3,6,18,19,42]. Briefly, hepatocytes were obtained from 180–210-g Sprague-Dawley rats by a two-step in situ collagenase perfusion method. The cellular DNA and protein content in the hepatocytes were measured simultaneously by two-parameter flow cytometry (sulphorhodamine 101 for protein and 4,6-diamidino-2-phenylindole dihydrochloride for DNA). The protocols used were previously evaluated with fibroblast-like cells[28,30]. Rat liver epithelial cells for co-culture experiments were isolated from 10-day-old Fischer (F344) male rats[3,51]. These cells most probably are of bile duct or of endothelial origin. Specific variation in the culture conditions are mentioned when necessary in the corresponding section in 'Results and discussion'.

Results and discussion

DNA and protein analysis. The analysis of ploidy and cellular protein content by flow cytometry proved to be a valuable tool in the characterization of hepatocyte populations. Histograms obtained after flow analysis of freshly isolated cells are shown in figure 1. Information includes a) the biological age of the animals from which hepatocytes were isolated or the senescence of the culture (relationship between the 2 N, 4 N and 8 N hepatocytes), b) the contribution of nonparenchymal cells (2 N-cells with low protein-content), c) the loss, accumulation or proliferation of cells of specific ploidy classes, d) alterations in the cellular protein content in individual ploidy classes (nuclei-cytoplasma relationship) and e) acute toxic events (particles with an unusually low protein or DNA content or particles with an abnormal relationship between protein and DNA content). Accurate DNA content measurements of freshly isolated and cultured rat hepatocytes were routinely obtained to an accuracy of 4–5% as shown by the coefficient of variations (CV) summarized in table 1. Protein values measured with the sulphorhodamine stain provided reliable relative estimates of the true cellular protein content (fig. 2). A close relationship was found between the position of the modus of the distribution of cellular proteins and the total protein content of the same cell population determined biochemically by Bradford's method[7].

Since the liver is involved in the regulation of nutrient concentration in the blood, hepatocytes must be able to rapidly change their rate of autophagy and lysosmal proteolysis. During starvation, protein degradation may provide organic substrates for essential metabolic processes. Cellular protein content, which includes the heterogeneous collection of enzymes and structural elements, is therefore an overall indicator of the metabolic state of cells. During undisturbed growth it is assumed that the ratio between DNA and protein is constant. This

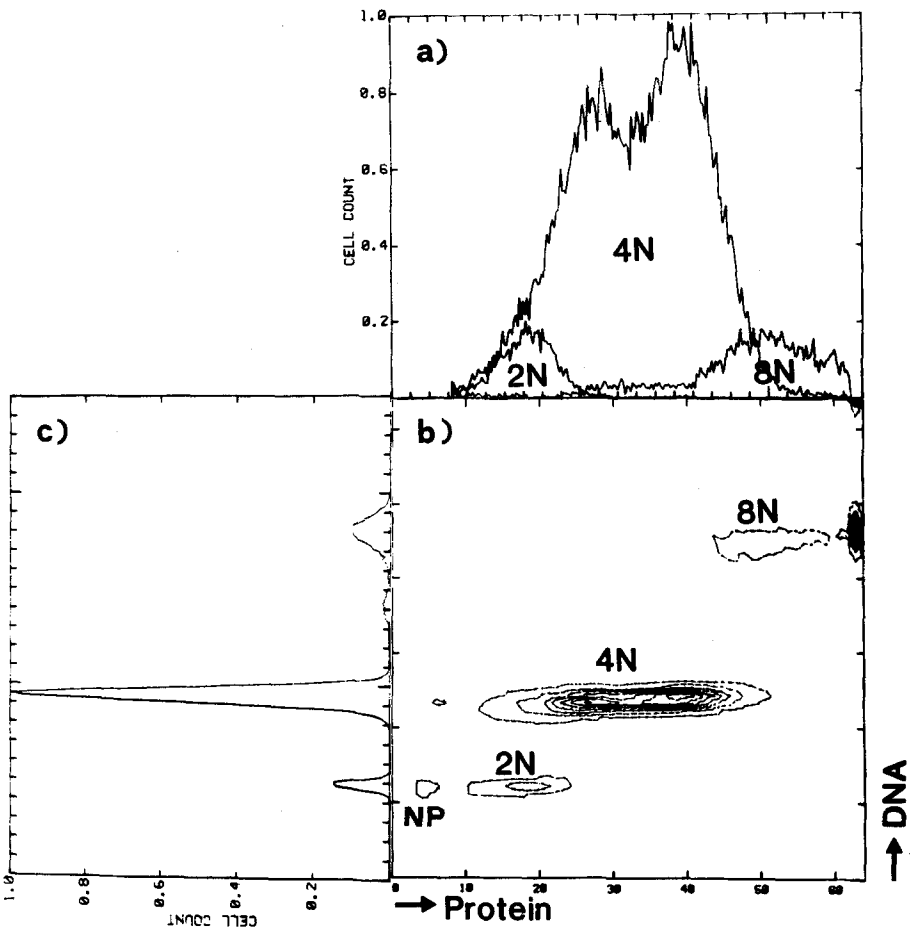

Figure 1. DNA and protein plots obtained after flow analysis of 5×10^5 freshly isolated hepatocytes from a 200-g male rat. Hepatocytes were enriched by low speed centrifugation (20 × g, 3 × in PBS). a) Protein distribution of individual ploidy classes; b) contour plots; c) DNA content. NP = nonparenchymal cells.

Table 1. Relative contribution of hepatocyte ploidy classes, coefficient of variation of DNA distribution, channel position of modus of the x-log normal distribution (average protein content) and width of distribution (heterogeneity) in untreated cultures. The relative alteration compared to freshly isolated hepatocytes, is shown separately for the low-protein (l.p.) and high-protein (h.p.) 4 N hepatocytes.

DNA	Ploidy (%)												Coefficient of variation							
	2N				4N				8N				2N				4N			
Culture time	0d	1d	4d	7d	0d	1d	4d	7d	0d	1d	4d	7d	0d	1d	4d	7d	0d	1d	4d	7d
mean	19.8	14.0	10.8	15.01	64.2	70.6	75.7	71.2	16.1	15.4	13.5	13.8	5.2	5.7	5.0	4.7	5.0	6.2	4.5	3.8
SD	±8.6	±8.3	±4.1	±3.5	±5.1	±9.0	±3.3	±2.7	±5.6	±3.9	±3.1	±2.4	±1.0	±1.7	±1.1	±1.2	±0.8	±2.3	±1.4	±0.7
%	100	71	55	76	100	110	118	111	100	96	84	86	100	109	96	91	100	124	89	76

Protein	Channel position									W-value								
	2N				4N					2N				4N				
Culture time	0d	1d	4d	7d	0d	0d	1d	4d	7d	0d	1d	4d	7d	0d	0d	1d	4d	7d
					l.p.	h.p.								l.p.	h.p.			
mean	97	56	55	54	134	225	114	100	87	1.53	1.66	1.61	1.7	1.69	1.32	1.56	1.41	1.53
SD	±25	±16	±18	±16	±27	±43	±23	±31	±27	±0.12	±0.14	±0.17	±0.13	±0.20	±0.20	±0.20	±0.10	±0.09
%	100	58	57	56	100		85	75	65	100	108	105	111	100		93	84	91
						100	51	44	39						100	118	107	116

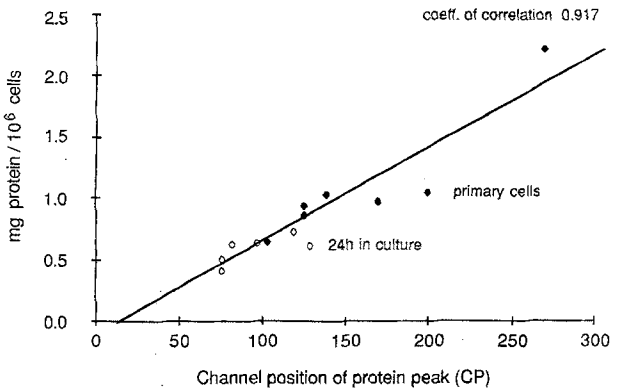

Figure 2. Relationship between protein content of 10^6 cells determined biochemically (Bradford 1976) and the channel position of the modus of the cellular protein distribution of the same 4 N cells after flow analysis.

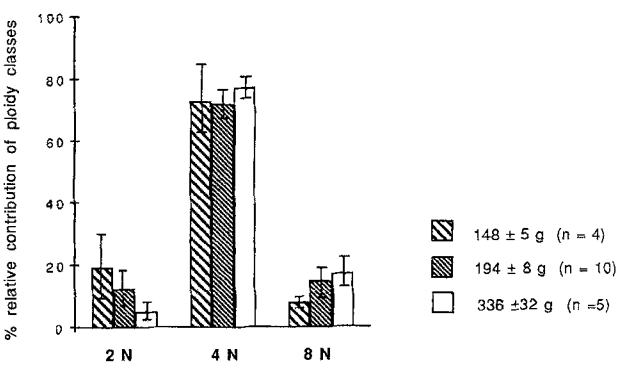

Figure 3. Contribution of hepatocyte ploidy classes after perfusion of male Sprague-Dawley rats of differing ages. Hepatocytes were enriched by low speed centrifugation.

was the case; the three ploidy classes of hepatocytes maintained a close nuclei-cytoplasm ratio (fig. 1 b).

Freshly isolated hepatocytes. The analysis of cells after liver perfusion allows the determination of the enrichment procedure during hepatocyte isolation, the biological age of the animal, the composition of the cells in cultures and effect of treatment to be monitored. In liver, 65% of the cells are of parenchymal origin and represent 90% of the total cell mass [49]. Therefore, after perfusion hepatoxytes are enriched either by low speed centrifugation (3×5 min at $20 \times$ g) or by sedimentation in culture medium for 15 min at room temperature. Flow analysis allowed the hepatocyte isolation procedure to be monitored. Enrichment of hepatocytes by low speed centrifugation or sedimentation and the batch of collagenase influenced the contribution of ploidy classes. After centrifugation, $15.3 \pm 5.2\%$ were 2 N cells, $66.7 \pm 4.4\%$ were 4 N and $18.0 \pm 2.2\%$ 8 N cells ($n = 5$). After sedimentation, the corresponding values were $24.2 \pm 9.6\%$, $61.7 \pm 4.7\%$ and $14.1 \pm 7.4\%$ ($n = 5$). No differences were found in the cellular protein content.

In rat liver, during aging of the animal, diploid 2 N-hepatocytes, include stem cells, develop into tetraploid (4 N, binucleated, mononucleated) and finally octoploid (8 N) hepatocytes. Ploidy shifts, therefore, could serve as indicators of animals age [21]. This was demonstrated for freshly isolated hepatocytes from rats of differing body weight and age (fig. 3). Clearly an age-dependent relationship between 2 N, 4 N and 8 N cell populations was found. In the case of the 4 N hepatocytes, two subpopulations with differing protein content are apparent (fig. 1 a). The contribution of the two 4 N-hepatocyte subpopulations varies with the age of the animals (fig. 4). The variability found between individual perfusions, and between hepatocyte enrichment procedures and collagenase batches indicates that hepatocyte cultures should be established from individually analyzed and defined cell populations. Drug-induced effects can thus be determined from parallel cultures and expressed as changes relative to the untreated cultures.

Hepatocytes in culture. Culture conditions affect very much the behavior of hepatocytes. Therefore conditions are mentioned briefly. We used a mixture of Modified Eagles Medium and Medium 199 (3:1, v/v), supplemented with gentamycin (50 µg/ml), insulin (10 µg/ml), 10% fetal calf serum during the 3-hour attachment period, and subsequently with 70 µM hydrocortisone. Dishes were coated with gelatin or collagen [3, 18, 19]. Alterations in the relative contribution of ploidy classes after culture for 1–7 days are summarized in table 1. The number of 2 N and 8 N hepatocytes decreased drastically and moderately respectively, whereas the number of 4 N cells increased by between 10 and 20%. The data indicate that the tetraploid hepatoxyte population is principally responsible for the metabolic capacity in cultures. According to the ploidy values in table 1, in culture, 2 N cells are preferentially lost up to day 4. This cell population probably includes stem cells, the survival of which may not be supported effectively in serum-free medium. Protein degradation takes place most heavily within the first 24 h

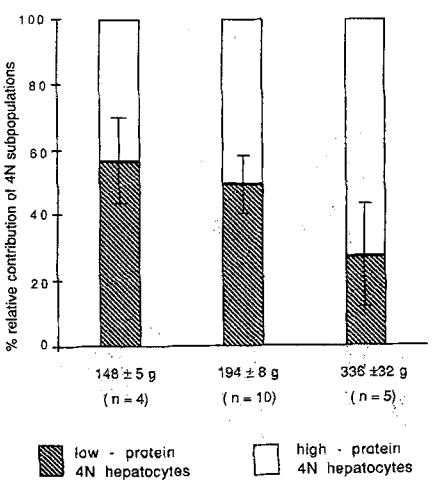

Figure 4. The contributions of subpopulations in freshly isolated 4 N hepatocytes. Number of cells are calculated in the two peaks (low and high protein) of the 4 N protein histogram and expressed as a percentage of the sum of the total number of 4 N hepatocytes.

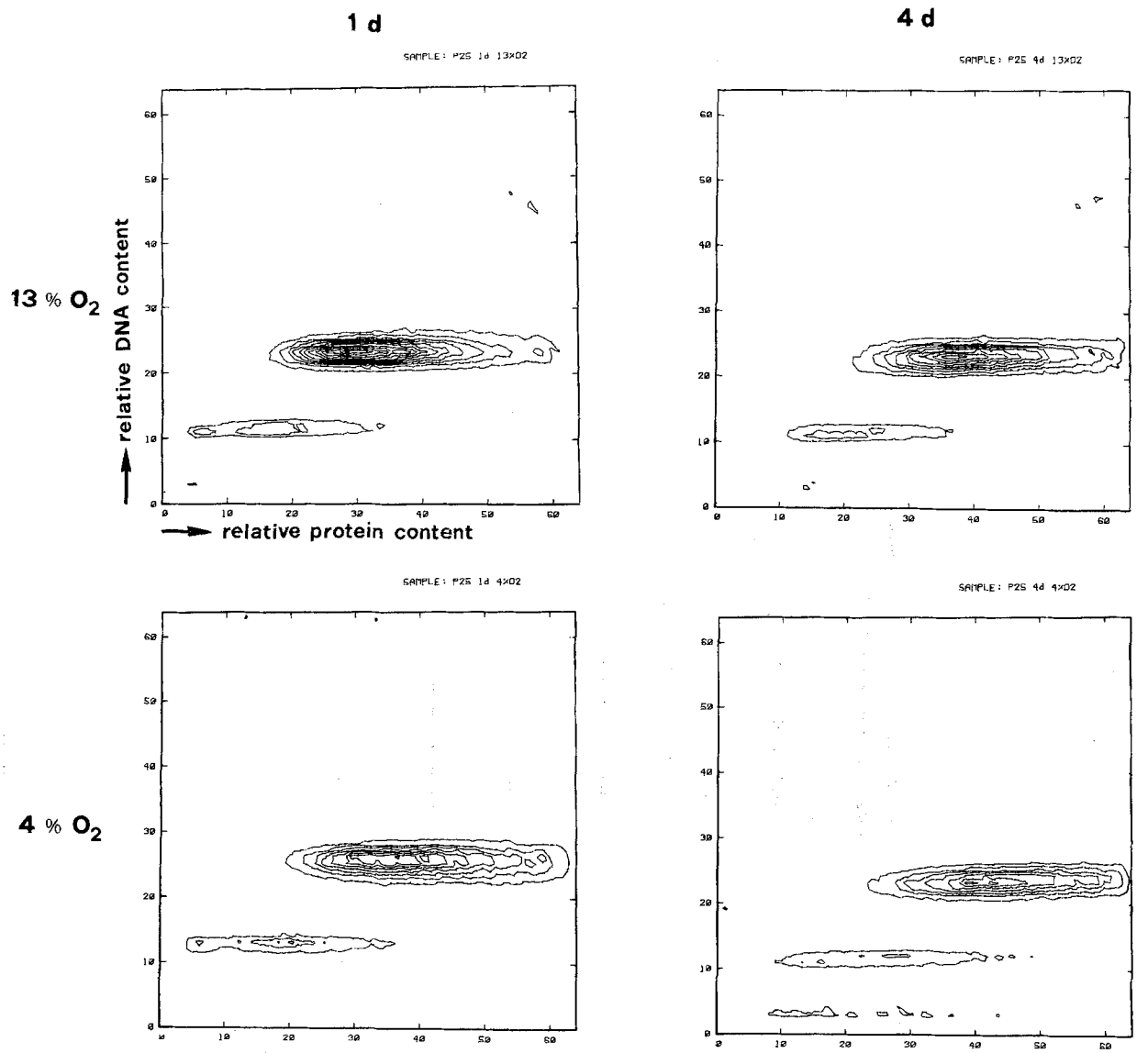

Figure 5. Contour plots of DNA/protein analyses of hepatocytes cultured for 1 and 4 days at periportal (13% O_2) and perivenous (4% O_2) oxygen tension. Note the shifts in protein.

(table 1). In hepatocyte cultures (monocultures), the available culture media probably fail to provide sufficient external nutrients; the rate of proteolysis in hepatocyte cultures may reach 5%/h[43]. It is concluded that the 4 N hepatocytes with high protein content preferentially lose their cellular protein during the first 24 h in culture, this degradation occurring less rapidly in the low-protein 4 N hepatocytes. By this point in time the peaks of the two cell populations in the protein histogram have merged into one (fig. 5). This agrees well with the concept of down regulation of cellular proteins to the optimum concentrations of macromolecules and organelles. This optimum seems to be similar for both the low- and high-protein hepatocytes, as further suggested by the decreasing w-value as an indicator of heterogeneity in the protein distribution of the 4 N hepatocytes (table 1). From this variation with times, it is suggested that the protein loss in high-protein 4 N hepatocytes parallels the degradation of enzymes involved in xenobiotic metabolism (fig. 8). Enzymes or isoenzymes specific to adult, differentiated hepatocytes disappear in culture more rapidly than enzymes which are present also in less differentiated cells such as intracellular LDH[3]. Accordingly, we conclude that enzymes present in the high-protein 4 N cell population which increased with age of the animal are subjected to a discriminate protein sequestion by autophagy and lysosomal proteolysis.

The increase in the 2 N population by day 7 (table 1) indicates that proliferation or preferential maintenance of undifferentiated 2 N cells might be taking place. Newly deriving cells cannot be separated unambiguously from 2 N hepatocytes because the latter's cellular protein content after 7 days in serum-free medium is only half the original value, and this is comparable to the value of

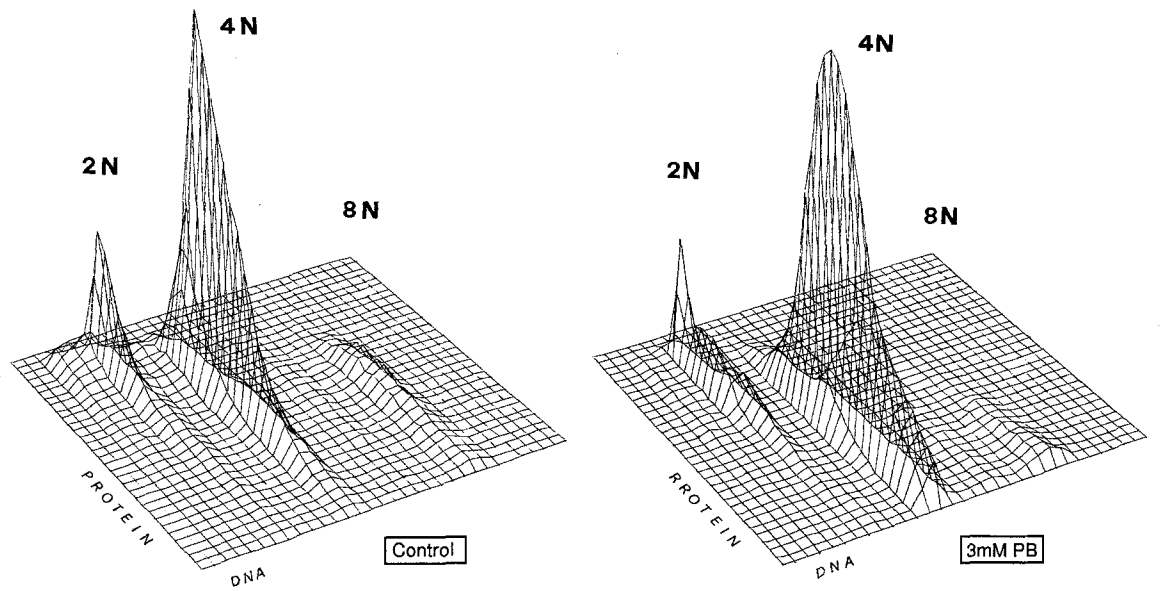

Figure 6. Three-dimensional graph of data obtained after exposure of hepatocytes to phenobarbital. After 7 days in culture, the relative decrease of the 2 N and the 8 N hepatocytes, increase of the 4 N cells and increase in cellular protein in all three ploidy classes are apparent.

Table 2. Relative contribution of hepatocyte ploidy classes (ploidy %) and average protein content (CP) after exposure to different modulators of cell differentiation.

	Ploidy (%)									Protein (CP)					
	2N			4N			8N			2N			4N		
Culture time	1d	4d	7d	1d	4d	7d	1d	4d	7d	1d	4d	7d	1d	4d	7d
DMSO 2% (n = 4)															
mean	10.0	9.1	9.6	74.9	75.6	74.2	15.2	15.4	16.1	61	64	64	110	117	91
SD	±3.3	±1.4	±4.0	±4.3	±2.6	±3.4	±1.3	±1.3	±1.2	±22	±17	±11	±43	±49	±23
Deviation from controls	0.5	−0.9	−5.6	−2.4	−0.4	2.5	2.7	1.3	2.5	2	0	−7	0	7	5
Δ%	105	91	63	97	99	103	122	109	119	103	100	91	100	107	106
4% O2 (n = 12)															
mean	10.6	12.3	23.6	77.9	75.3	67.8	11.5	12.3	8.7	65	58	50	122	98	72
SD	±2.5	±4.4	±10.7	±3.7	±5.0	±7.3	±2.5	±2.8	±4.9	±15	±19	±13	±31	±36	±20
sign.		***						***	*		*			*	
Deviation from controls	0.6	2.9	4.6	0.4	0.2	0.0	−1.9	−3.1	−4.6	−5	10	0	3	13	5
Δ%	106	130	124	101	100	100	86	80	65	93	120	100	103	116	108
10% FCS (n = 5)															
mean	27.3	22.7	24.2	59.7	67.8	64.8	13.0	9.5	11.0	41	52	51	110	106	111
SD	±9.9	±10.0	±7.9	±7.4	±7.2	±10.8	±4.0	±3.9	±3.2	±3	±7	±5	±23	±5	±40
sign.			*					*							**
Deviation from controls	7.5	9.6	6.9	−3.3	−6.9	−4.5	−4.3	−2.7	−2.3	4	4	−6	8	14	20
Δ%	138	173	140	95	91	93	75	78	83	109	108	89	108	115	122
Phenobarbital 3 mM (n = 7)															
mean	8.5	0.2	8.4	74.4	78.8	80.5	17.3	12.0	11.8	59	80	58	126	153	109
SD	±3.3	±5.2	±2.7	±4.6	±3.7	±4.8	±3.7	±2.3	±1.6	±17	±23	±21	±33	±39	±27
sign.		*	***		*	***	**				*			***	***
Deviation from controls	−0.6	−1.7	−4.7	−1.3	3.3	7.2	2.4	−1.6	−3.1	1	20	8	10	45	35
Δ%	93	84	64	98	104	110	116	88	79	102	132	116	108	142	147

sign. paired t-test; * $p > 0.01$; ** $p > 0.01$; *** $p > 0.001$.

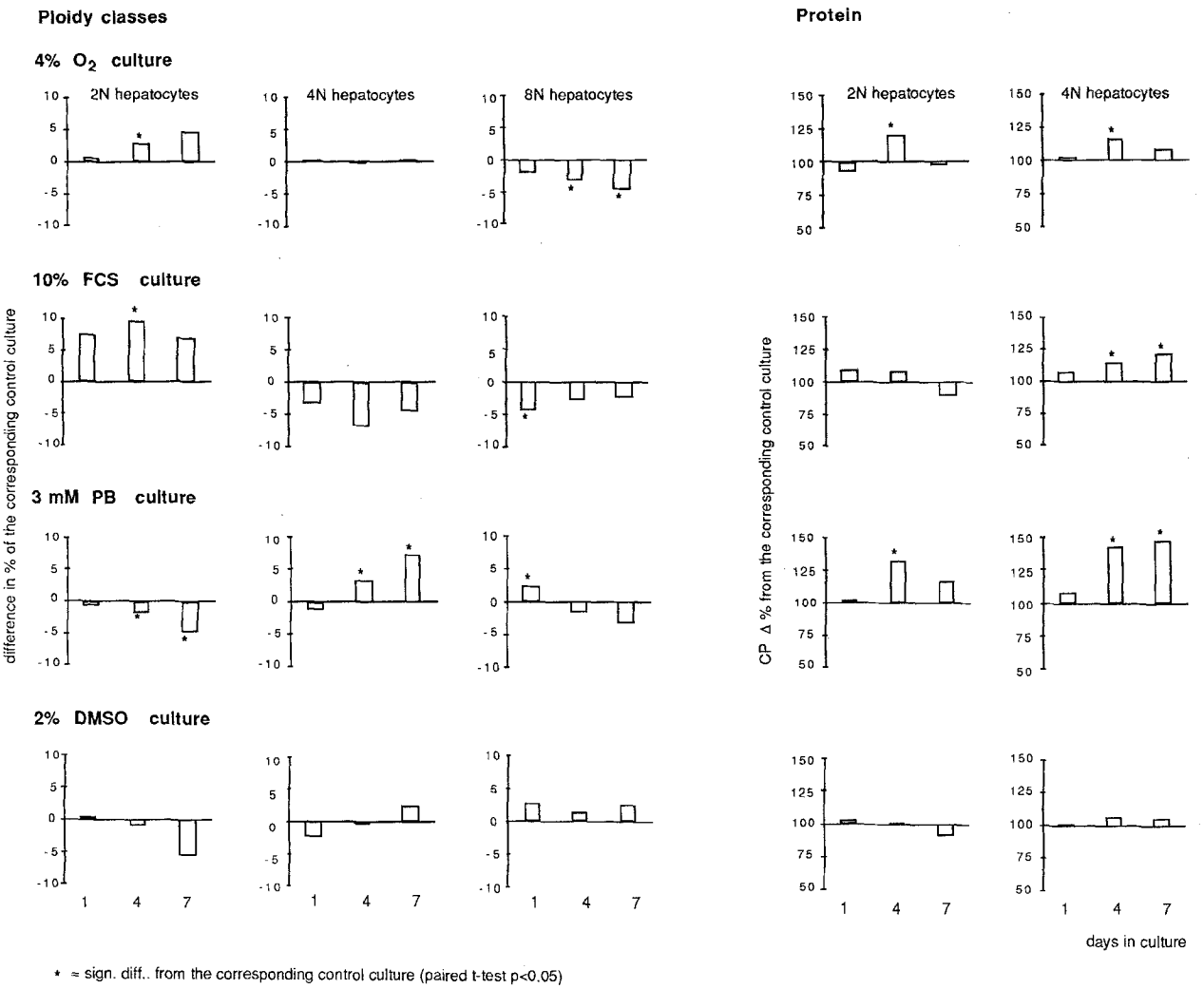

Figure 7. Ploidy-shifts, and alterations in cellular protein content after exposure of hepatocytes to DMSO, PB, 4% O_2 and 10% FCS. Data are expressed as differences relative to parallel-cultured untreated cells.

nonparenchymal cells (fig. 1 b). In conclusion, alterations in cellular DNA and protein content could provide suitable indicators of the metabolic state before and after treatment with chemicals.

Ploidy shifts. Aging of hepatocytes is probably controlled by differentiation processes. Therefore test compounds which affect ploidization, probably do so by interfering with differentiation processes in liver. After treatment of rats with tumor promoting agents or with carcinogens, alterations in the contribution of nuclei at different ploidy levels were observed [10, 36, 40, 45, 48]. Furthermore, the appearance of a diploid subpopulation in rats was detected within 8 weeks of carcinogen treatment [41]. Similarly, in vitro non-physiological alterations in ploidization of intact cells could be used as indicators of chemicals which pathologically affect differentiation in liver tissue, such as liver tumor promoting agents [26].

Data from all four modulators as a function of culture time, are summarized in table 2 and in figure 7. Data of 2% v/v dimethyl sulfoxide (DMSO) and 4% O_2 exposure were deduced from experiments published earlier. Each of the four induced a specific time-dependent shift in ploidy of 2 N, 4 N and 8 N cells. The results correspond well with the known biological activity of the agents:

Dimethylsulfoxide: In vivo, DMSO protects tissues against damages from oxygen radical attack [35, 46]. In vitro, the dipolar aprotic solvent induces the differentiation of transformed cells [9, 11, 20, 22, 50] and maintains the metabolism of differentiated cells such as adipocytes [50] or hepatocytes [20]. This activity agrees with shifts in ploidy observed in the experiments to levels found in adult rats and with the expression of a well-differentiated phenotype in culture [19].

Oxygen tension: When hepatocytes are seeded in densities sufficient to form a monolayer and cultured in an incubator atmosphere of 13% or 4% O_2 in plastic dishes, the majority of cells maintain their integrity determined

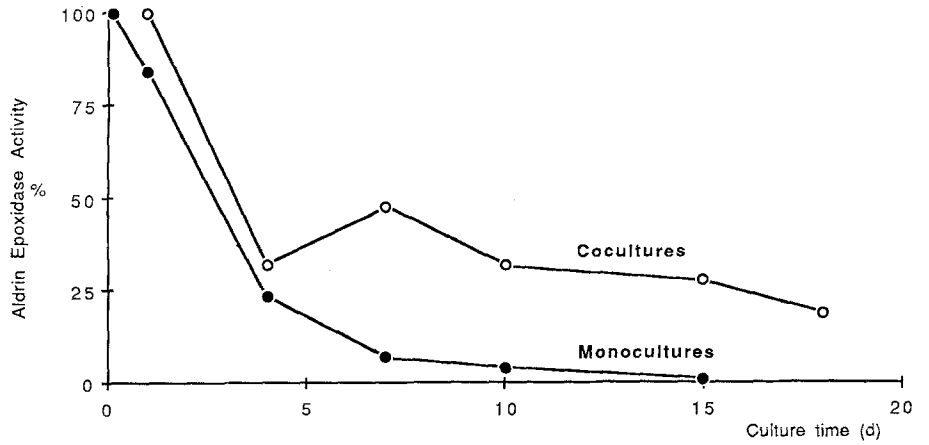

Figure 8. Stabilization of the aldrin epoxidase activity in co-cultures of hepatocytes with rat liver epithelial cells (RLEC). Activity is expressed as the percentage activity per plate of hepatocytes after 3 h (monocultures) or 24 h (co-cultures).

by cellular protein content within 1–2 days after seeding, but contain partially degraded nuclei (at 4% O_2 > 90%). Oxygen tension, measured in the medium above the monolayer, showed that oxygen depletion was a function of cell density in culture dishes.The seeding density chosen in the hepatocyte cultures with DMSO, PB and FCS, creates an oxygen tension comparable to that in the periportal area in liver. A stable, incubator-controlled low oxygen tension in the culture medium, independent of cell density, can only be maintained in petri dishes with teflon membranes [18]. Therefore, the influence of low tissue oxygen tension on ploidy was determined from teflon-membrane, collagen-coated culture dishes incubated in parallel at 13% and 4% O_2, respectively. Representative examples are shown in contour plots in figure 5.

In contrast to cultures with DMSO at 4% O_2 the ploidy shifts resemble those found in young animals. It seems that, the perivenous oxygen tension prevents the aging of hepatocytes in vitro [18]. This interpretation is supported by reports that low pO_2 stimulates in vitro growth of tumor cells [44] and of stem cells from bone marrow [16], and that low pO_2 prevents aging of cultured human cells [33]. It would seem that nuclear metabolism (release of endonucleases) is specifically sensitive to oxygen tension and that low oxygen tension delays constitutive differentiation processes.

Fetal calf serum: In the experiments with fetal calf serum (FCS) a batch of FCS which had been selected for maximal clonal growth of fibroblast-like cells was used and contains an efficient mixture of growth factors [17]. The preferential increase of 2 N cells observed might derive from the proliferation of undifferentiated stem cells which respond efficiently to growth factors.

Phenobarbital: In vivo the barbiturate PB has been reported to induce a hypertrophic and hyperplastic response in liver [38] and to promote hepatic tumors [38]. The compound alone can also be carcinogenic [37] and postpones cell death (apoptosis) [8]. After chronic exposure to 3 mM phenobarbital (PB), the ploidy shifts obtained in cultures demonstrate that PB blocks hepatocyte differentiation at the 4 N level. In figure 6, a representative set of data is presented as a three-dimensional projection. In accordance with the observed hypertrophic reaction of liver in vivo, the cellular protein content increased in all ploidy classes (fig. 6 and table 2). It is possible that this ploidy shift induced by PB could be a typical response of hepatocytes to liver-specific tumor promoters. More data are required to substantiate this interpretation.

Co-culture experiments. From a well-growing clone in vitro of rat liver epithelial cells a cell line was established and used between passage 10 and 13 [3]. For co-cultures, the auxiliary cells were added after the 3-hour attachment period (1×10^6 hepatocytes, 2×10^6 auxiliary cells in uncoated 60-mm dishes), and the morphology of hepatocytes, the daily acute toxicity (cell detachment), cellular protein content, intracellular lactate dehydrogenase activity and conversion of aldrin to dieldrin were examined simultaneously [3]. The method used to determine aldrine epoxidase activity allows picograms of produced dieldrin to be detected [25]. Cultures were maintained for up to two weeks. Unlike the monocultures of hepatocytes, the co-cultured hepatocytes expressed a differentiated phenotype, bile canaliculi were prominent, no cell detachment was detectable and proteins and lactate dehydrogenase (examples of nonspecific parameters) were completely stabilized over the whole culture period. In monocultures the LDH-activity decreased rapidly within 1–2 days. Aldrin epoxidase, a cytochrome P-450, phenobarbital inducible isoenzyme, was maintained in co-cultures for more than 14 days at approximately 40% of the activity found after 3 h in culture (fig. 8). The auxiliary cells showed no significant activity [3]. Therefore this conversion of aldrin to dieldrin can be fully attributed to the liver cells. The presence of another specific cell type inhibits proteolysis of an enzyme involved in xenobiotic metabolism drastically. This effect cannot be attributed to external nutrient supply: stabilizing factor(s) exchanged between hepatocytes and biliary cells by a direct

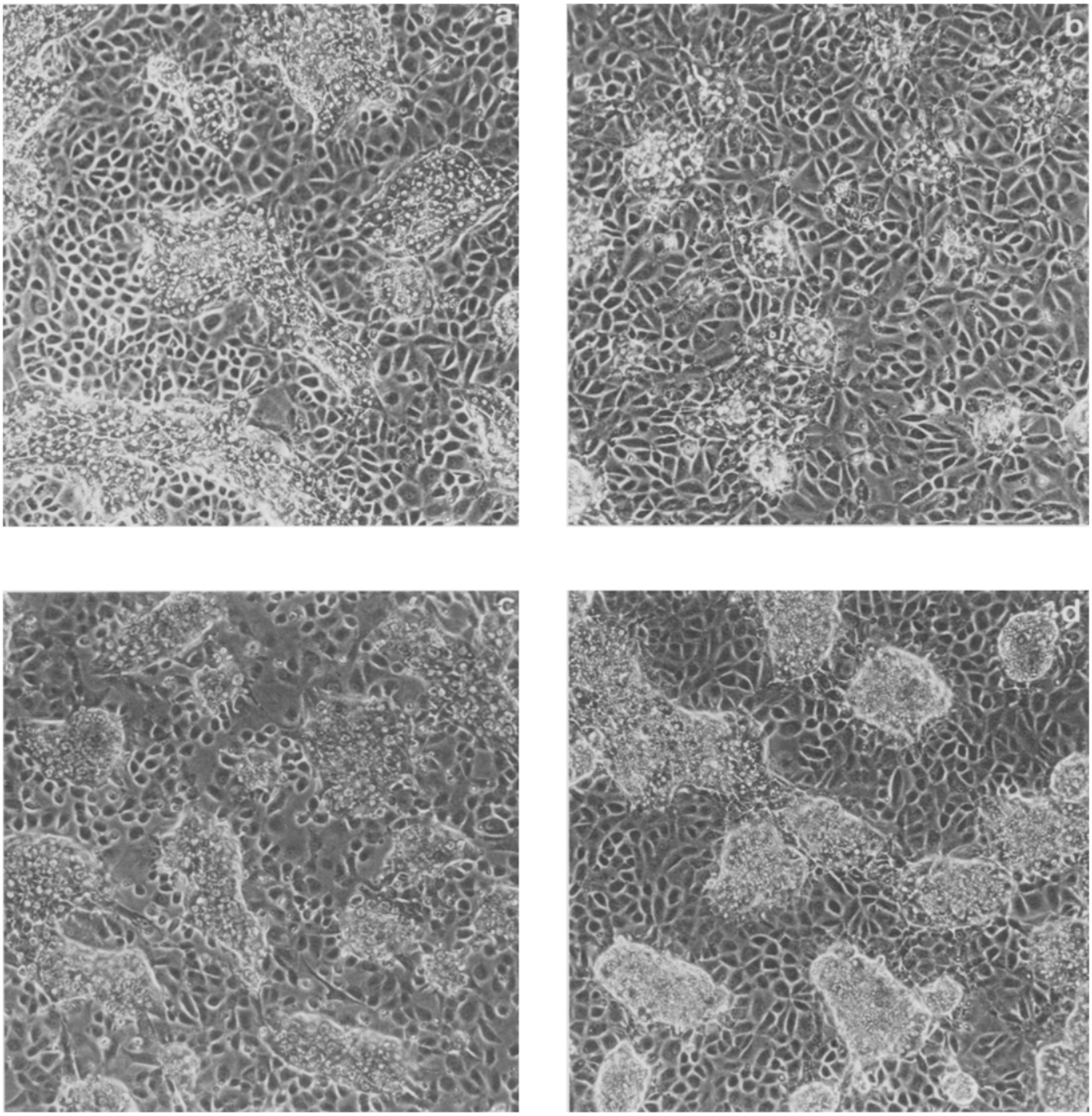

Figure 9. Morphology of hepatocytes and auxiliary cells cultured for 4 days, 48 h after exposure to selected chemicals. a) control; b) 2-AAF; c) procarbazine; d) cyproterone-acetate.

cell-cell communication must be postulated. The decrease within the first four days may be a result of the time period necessary to establish functional junctions between the two cell types. Further investigations are necessary to determine whether phase I and phase II reactions are balanced in these hepatocytes and whether other hepatocyte-specific functions are also stabilized.

The toxicities of selected chemicals in co-cultures of hepatocytes with RLEC were determined in preliminary experiments. Three compounds whose pharmacokinetic behavior has been well described were chosen, including 2-acetylaminofluorene (2-AAF) as a hepatotoxic liver carcinogen, procarbazine which exerts a mutagenic[27] and carcinogenic activity in most extrahepatic tissues[53] and is converted in liver to stable intermediates, and cyproterone acetate, the gestagenic steroid, as an example of an agent which induces adaptive growth in liver[39]. Figure 9 shows representative morphologies of cells at day 4, 48 h after exposure. The three classes of chemicals can be distinguished by the differing patterns of damage they cause to co-cultured auxiliary cells and hepatocytes. 2-AAF selectively killed the hepatocytes without affecting the RLEC (fig. 9 b); procarbazine at high doses was preferentially toxic to RLEC (fig. 9 c) cells and cyprote-

rone acetate specifically stimulated growth of RLEC. Auxiliary cells tended to overgrow the hepatocytes (fig. 9 d). Further combined tests, measuring biochemical variables such as albumin, protein and cholesterol syntheses and aldrine epoxidase, are necessary to characterize more precisely the response of cocultures in toxicity testing. However, the observation of selective toxicity in the two populations for each of the three chemicals, 2-AAF, procarbazine and cyproterone-acetate demonstrates that heterotypic hepatocyte cultures increase the spectrum of toxic and subtoxic events. Accordingly, this approach will detect a broader spectrum of hepatotoxic agents and provide more reliable data than monocultures.

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Use of aggregating cell cultures for toxicological studies

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Summary. Relatively simple techniques are now available which allow the preparation of large quantities of highly reproducible aggregate cultures from fetal rat brain or liver cells, and to grow them in a chemically defined medium. Since these cultures exhibit extensive histotypic cellular reorganization and maturation, they offer unique possibilities for developmental studies. Therefore, the purpose of the present study was to investigate the usefulness of these cultures in developmental toxicology. Aggregating brain cell cultures were exposed at different developmental stages to model drugs (i.e., antimitotic, neurotoxic, and teratogenic agents) and assayed for their responsiveness by measuring a set of biochemical parameters (i.e., total protein and DNA content, cell type-specific enzyme activities) which permit a monitoring of cellular growth and maturation. It was found that each test compound elicited a distinct, dose-dependent response pattern, which may ultimately serve to screen and classify toxic drugs by using mechanistic criteria. In addition, it could be shown that aggregating liver cell cultures are capable of toxic drug activation, and that they can be used in co-culture with brain cell aggregates, providing a potential model for complementary toxicological and metabolic studies.
Key words. Aggregating cell cultures; brain cell cultures; liver cell cultures; teratogenesis; toxicology; antimitotic drugs; cholera toxin.

Introduction

The technique of rotation-mediated aggregating cell culture has been introduced by Moscona [26, 27], who showed that freshly isolated immature cells of any fetal organ are able to reassociate spontaneously in vitro, giving rise to three-dimensional, organotypic cultures. Subsequently, this culture system has been applied mainly for developmental studies of the brain. These investigations showed on morphological as well as on biochemical grounds that aggregating fetal brain cells are able to mimic several morphogenetic events occurring in vivo (e.g., cell migra-