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THE EFFECT OF PHENOBARBITAL ON PROTEIN METABOLISM OF LIVER. RESULTS WITH ISOLATED HEPATOCYTES

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Abstract—A technique has been devised which makes use of an amino acid alternatively labeled with either [^{14}C] or [^{3}H], and permits the *simultaneous* evaluation of synthesis, catabolism and secretion by the same sample of isolated rat liver cells during the same time-period of incubation. This technique has been used to study protein metabolism of liver cells isolated from rats treated with 4 doses of phenobarbital (8 mg/100 g body weight) given in the 4 days before killing the animals. Total protein synthesis and secretion do not change in phenobarbital-treated rats; albumin represents 40% of secreted protein in both normal and treated rats. On the contrary, the parameters which indicate protein degradation are lower in phenobarbital-treated than in normal rats, showing that protein catabolism is appreciably reduced in the liver cells obtained from rats treated with phenobarbital.

Cell growth depends on accumulation of protein, that may or may not be accompanied by alterations in the relative proportions of the single protein species. This accumulation may be reached through increase in synthesis, decrease in degradation, or a combination of both mechanisms. Recent results emphasize the importance of decreased degradation in some growth processes such as liver regeneration [1] and the early stages of postnatal liver development [2]. A third less usual mechanism, which can operate synchronously to those quoted above, is represented by the intracellular storage of proteins normally destined to secretion, and has been described in liver tumors [3, 4].

Distinct pathways are used for the synthesis and degradation of proteins, thus creating a metabolic cycle with the possibility of fine control mechanisms at different steps; detailed but separate studies of each of these pathways may often overlook the entire picture of events, sometimes for purely technical reasons. For instance, the use of a single isotope does not allow a comprehensive evaluation of synthesis, catabolism and secretion at the same time.

Continuing our investigation on the regulation of protein synthesis in growing cells [5, 6] we have now chosen to study the effects of phenobarbital (PB), a well-known inducer of drug metabolizing enzymes, which causes liver hypertrophy, stimulates protein synthesis, increases the number of polyribosomes [7] and, after some negative results, [8, 9] has recently been shown to increase the transcriptional activity of isolated liver nuclei [10].

To carry out the present research work we have devised and applied a technique which overcomes the difficulties discussed above by making use of an amino acid alternatively labelled with either [^{14}C] or [^{3}H], and permits the simultaneous evaluation of synthesis, catabolism and secretion by the same isolated rat liver cells during the same time-period of incubation.

MATERIALS AND METHODS

Chemicals. Collagenase (Clostridiopeptidase A), specific activity: *ca* 0.15 U/mg, and Cycloheximide were obtained from Boehringer GmbH, Mannheim (F.R.G.). L-[U-^{14}C]Leucine (342 mCi/mmole) and L-[4,5-^{3}H]leucine (132 Ci/mmole) were from the Radiochemical Centre, Amersham (U.K.). The scintillator (Omnifluor) was purchased from NEN Chemicals GmbH, Dreieichenhain (F.R.G.). All of the other chemicals and solvents, of reagent grade purity, were obtained from commercial sources.

Animals. Male albino rats (Wistar strain) weighing 200–250 g, maintained on a balanced diet of laboratory chow (Ditta Piccioni, Brescia, Italy) and kept under a standardized light–dark cycle (light from 07:00 to 19:00 hr) were used throughout. Phenobarbital (20 mg/ml in 0.154 M NaCl) was administered by intraperitoneal injection in 4 doses of 8 mg/100 g body weight [11] each every 24 hr. Food and water were provided *ab libitum*. The rats were killed between 09:00 and 10:00 hr, 24 hr after the last injection.

Isolation of hepatocytes. Rats were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally) and *in situ* liver perfusion was performed according to the method of Berry and Friend [12] with few modifications: the livers perfused with Ca^{2+} free Krebs–Henseleit saline [13] containing 0.035% collagenase, at a perfusion rate of 15–20 ml/min under a gas phase of 95% O_2 + 5% CO_2. After 20–25 min the liver was excised, minced and incubated with the enzyme-containing medium at 37° in a

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briskly moving Dubnoff-type shaker for 10 min. The disrupted liver was then filtered through nylon mesh (ϕ 400 μm) and centrifuged at 50 g (r_{max} 15 cm) for 2 min. The pellet was washed twice with Krebs-Henseleit saline containing a mixture of 20 L-amino acids at 5 times the concentration found in rat serum [14]. The hepatocytes were finally suspended in this medium (medium A) at a density of 10×10^6 cells/ml. Cell viability was assessed by the trypan blue exclusion test: all the preparations used in the present experiments had a viability of 90% or better.

Measurement of protein turnover. Leucine is an appropriate choice in studies on protein turnover [15, 16] in particular when high concentrations (0.85 mM) of unlabeled leucine, plus a mixture of 19 other amino acids at five times their plasma concentration, are used with the labeled molecule; under these conditions reutilization of isotope is minimized [17] and possible dilution effects due to the total intracellular leucine pool are largely prevented [18].

The experiment consists of two distinct parts: (i) a preliminary phase, carried out in the presence of [^{14}C]-leucine, during which the proteins of the hepatocytes become progressively labeled with [^{14}C], (ii) the actual phase of measurements, carried out in the presence of [^{3}H]-leucine, during which the intracellular proteins loose their TCA-precipitable [^{14}C]-label (protein catabolism) and increase their TCA-precipitable [^{3}H]-label (protein synthesis): at the same time TCA-precipitable [^{14}C]-radioactivity appears in the incubation medium (secretion of proteins synthetized during the preliminary phase of the experiments = early proteins). In the last time-period of this incubation some TCA-precipitable [^{3}H]-radioactivity also appears in the medium (secretion of proteins synthetized during the second phase of the experiment (= late proteins).

(i) Pre-labeling of hepatocyte protein with [^{14}C]-leucine: 2.5 ml of cellular suspension were incubated at 37° for 60 min in 25 ml conical siliconized glass flasks in the presence of L-U-[^{14}C]-leucine (5 μCi) and in an atmosphere of 95% O_2 + 5% CO_2. Incubation was stopped by dilution with 7 ml of Medium A maintained at room temperature. The cells were centrifuged at 25 g for 5 min, the radioactive supernatant was discarded and the cell pellet washed twice with Medium A in order to remove extracellular [^{14}C]-leucine. The intracellular non incorporated labeled leucine was removed by re-incubating the cells for 5 min in 10 ml isotope-free Medium A. The cells were sedimented at 25 g for 4 min and finally resuspended in 10 ml fresh Medium A. Cell protein was determined by a standard biuret method [19]. Rat serum albumin was used as reference standard.

(ii) Incubation with [^{3}H]-leucine: [^{14}C]-labeled cells were incubated in 1.5 ml of Medium A containing 15 μCi L-4.5[^{3}H]-leucine. Incubation was carried out at 37° in an atmosphere of 95% O_2 + 5% CO_2. At fixed times (0, 15, 30, 45, 60 min) incubation was stopped by addition of 150 μl 1 mM cycloheximide. Samples were immediately centrifuged at 60 g for 4 min. The resulting supernatant was collected for the determination of radioactivity bound to secreted protein. The cell pellet was resuspended in 1.5 ml of a medium containing 150 mM-NaCl, 20 mM Tris–HCl, pH 7.4, 100 μM cycloheximide, and processed for the determination of intracellular radioactivity as described.

Determination of radioactivity incorporated into protein. The preparation of samples for counting varied according to the source of material: (i) The cell pellet was resuspended in 1.5 ml 150 mM–NaCl; 20 mM Tris–HCl, pH 7.4; 100 μM cycloheximide. The suspension was adjusted to a final deoxycholate concentration of 0.65% (w/v) which was enough for maximal yield of soluble radioactive products. After centrifugation at 145000 g for 30 min at 20° the resulting pellet was discarded and the clear supernatant was collected for the determination of intracellular radioactivity. (ii) Aliquots of 250 μl of cell supernatant were used for the measure of the total protein secreted into the incubation medium. Further aliquots of 250 μl, supplemented with 250 μg of carrier rat serum albumin prepared by the method of Debro [20], were used for the determination of radioactivity in the secreted albumin according to the acid–alcohol procedure described by Scornik and Botbol [1].

All the samples were further processed in the same way: after addition of 2 ml of 1 N NaOH and incubation at 37° for 15 min the proteins were precipitated with 1 ml of 50% trichloroacetic acid (TCA), collected by filtration on glass fiber filters, washed with 10 ml 5% TCA and 10 ml of methanol/ether/chloroform (1, 1, 2, v/v), dried and counted in 10 ml of scintillation fluid (4 g Omnifluor/l of toluene).

Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (model 3365 Packard). In case where protein contained only [^{14}C]-leucine the efficiency of counting was 65%; when protein contained both [^{3}H] and [^{14}C] isotopes, windows were employed in which the [^{3}H] channel contained 4.5% of [^{14}C] counts, and the [^{14}C] channel contained no [^{3}H] counts. Efficiencies were of 19.1% for [^{3}H] and 33.8% for [^{14}C]. The standard error was 5% or better. No differential quenching among samples of the same type was encountered.

RESULTS

Preliminary experiments and calibration of the system. Protein content was chosen as standard reference basis of the metabolic activities of isolated liver cells mainly because it can be determined easily, quickly and with great sensitivity in minute amounts of cells. Results can also be related to the DNA content of the samples considering that the amount of protein in the normal liver cell is 111.63 ± 3.34 mg/mg DNA, and this value increases significantly to 142.04 ± 6.55 in phenobarbital treated rats (means of 5 experiments ± S.E.M.; $P < 0.05$). It should also be noticed that the amounts of protein metabolized by the cells are indicated as the molecules of protein leucine (i.e. TCA-precipitable leucine) calculated on the basis of the specific activity of the labeled precursor present in the incubation medium.

The metabolic capacity of our preparation of isolated hepatocytes has been assessed in some preliminary experiments. The incorporation of [^{14}C]-leucine into protein proceeds linearly for at least 3 h, and is entirely prevented by anaerobiosis (i.e., incu-

bation under nitrogen); therefore, the study of the metabolism of our cells can be safely carried out within this time-period.

When liver cells, previously incubated for 60 min in the presence of [^{14}C]-leucine, are re-incubated in a medium which does not contain this labeled molecule, there are both a progressive decrease of protein-associated, intracellular radioactivity and a progressive increase of [^{14}C]-labeled protein in the incubation medium. When the re-incubation in the absence of labeled leucine is performed anaerobically, the decrease of intracellular protein radioactivity is strongly reduced and the appearance of labeled protein in the medium is entirely suppressed by the 15th min of re-incubation. This shows that the release of TCA-precipitable radioactivity to the medium is an energy-dependent process and represents true secretion rather than flaking or partial destruction of the cells.

Protein synthesis, secretion and catabolism in isolated liver cells from PB-treated rats. The study was performed over a time-period of 1 h with cells previously labeled with [^{14}C]-leucine (preincubation) and then incubated in a medium containing [^{3}H]-leucine as a tracer. The results are illustrated in the following figures, which describe different aspects of the same experiments. Figure 1(A) shows that the intracellular proteins synthesized during the preincubation are progressively lost from the cells during the incubation. PB-treated and control cells have two different initial levels of [^{14}C]-labeling, reached at the end of the first part of the experiments: however, this difference is not statistically significant.

Figure 1(B) shows the secretion of [^{14}C]-labeled proteins into the medium; these proteins ("early proteins") represent a consistent amount, but not all of the proteins lost from the cells as described above. Liver cells from PB-treated rats do not show a rate of secretion appreciably different from the controls. Figure 1(B) describes protein secretion in bulk. Figure 1(C) shows the secretion of a defined protein, liver albumin, labeled during the preincubation phase of the experiments.

In agreement with the data reported above albumin secretion does not change after PB treatment.

All the results presented above illustrate the

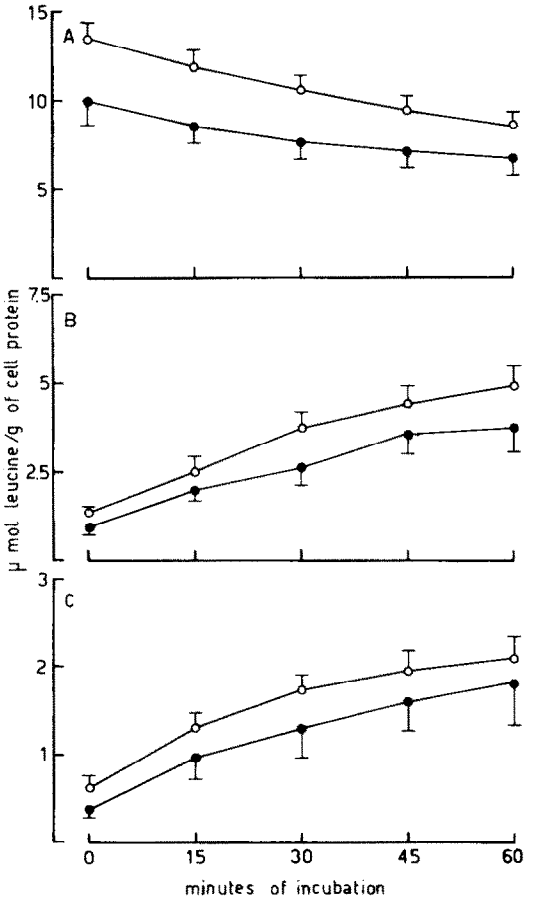

Fig. 1. Metabolism of [^{14}C] labeled proteins synthesized during the preincubation phase of the experiment. [^{14}C] pre-labeled cells were incubated at 37° in the presence of [^{3}H] leucine as described in Materials and Methods. ○ Normal cells, ● PB-treated cells, (A) loss of intracellular [^{14}C] labeled protein, (B) secretion of [^{14}C] labeled protein, (C) secretion of [^{14}C] labeled albumin.

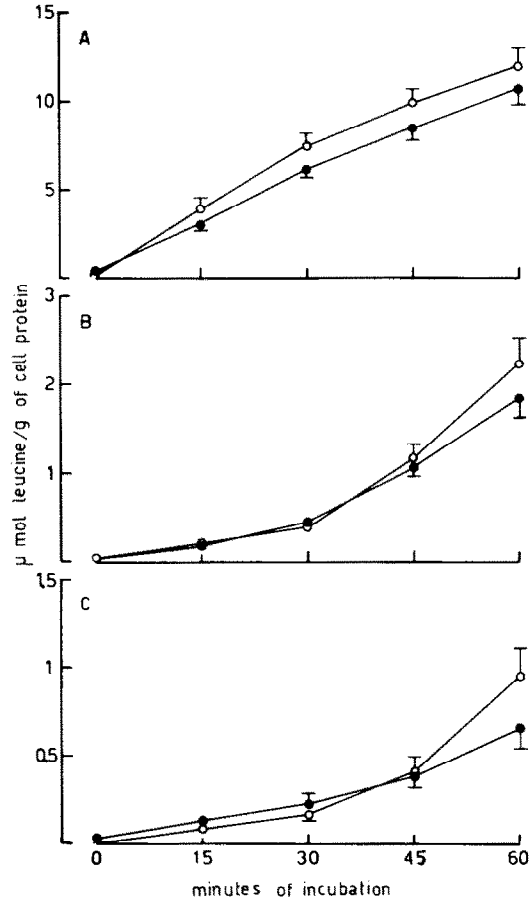

Fig. 2. Metabolism of [^{3}H] labeled proteins being synthesized during the second phase of the experiment. [^{14}C] pre-labeled cells were incubated at 37° in the presence of [^{3}H] leucine as described in Materials and Methods. ○ Normal cells, ● PB-treated cells, (A) synthesis of intracellular [^{3}H] labeled proteins, (B) secretion of [^{3}H] labeled proteins, (C) secretion of [^{3}H] labeled albumin.

metabolism (catabolism as well as secretion) of those proteins which become labeled with [^{14}C]-leucine during the pre-incubation phase of the experiment, and which we have defined as "early proteins".

The presence in the incubation medium of a new marker, i.e. [^{3}H]-leucine, allows the simultaneous study of protein synthesis, occurring synchronously with protein degradation and secretion. Figure 2(A) illustrates the synthesis of new intracellular protein and shows that synthetic activity is not significantly different in normal liver cells and in those obtained from PB-treated rats. The labeling of these cells reaches a level which is in the same range as the one obtained in the part of the experiment performed with [^{14}C]-leucine. Figures 2(B) and (C) describe the secretion of total protein and albumin synthesized during the incubation in the presence of [^{3}H]-leucine ("late proteins"). The secretion of [^{3}H]-protein occurs only after a consistent labeling of these protein with [^{3}H]-leucine thus explaining the lag phase of 20–30 min: even when accumulation begins to occur, the values of radioactivity at any single point of the experiment are not statistically different in PB-treated and control cells.

In Table 1 we give the balance sheet of the leucine contained in protein molecules which is metabolized during incubation. The data of the table allow the calculation of the amount of protein synthesized, secreted and possibly degraded, assuming that leucine represents 10.3% by weight of total protein [21] and 11.4% by weight of albumin [22]: therefore, 1 μmole of leucine, of which we know the specific activity, corresponds to 1270 μg of total protein and 1150 μg of albumin. On the basis of the data of Table 1 we draw the following conclusions: (1) The loss of intracellular proteins labeled with [^{14}C] is lower than normal in PB-treated rats. (2) Total protein secretion, including both [^{14}C]-labeled (early proteins) and [^{3}H]-labeled proteins (late proteins) is not significantly reduced in liver cells obtained from PB-treated rats. (3) Albumin secretion represents about 40% of total protein secretion in normal isolated liver cells; this proportion does not change in PB-treated rats. (4) Part of the radioactivity lost from intracellular protein is not found in the secreted protein. This apparent discrepancy is explained by release of labeled leucine as a free amino acid, and represents protein catabolism. This value is lower in PB-treated than in control rats, and shows that protein catabolism is appreciably reduced in the liver cells obtained from rats treated with PB. (5) Synthesis of intracellular protein during the time-period of incubation does not change in PB-treated rats.

Table 1. Balance sheet of the proteins metabolized during 60 min of incubation

	Loss of cell protein	Secretion of total protein	Secretion of albumin	Protein degradation	Protein synthesis
Control (6)	5.026 ± 0.33(a)	3.496 ± 0.48(a)	1.446 ± 0.15 (a)	1.530 ± 0.33 (a)	11.754 ± 1.01(b)
		2.314 ± 0.29(b)	0.904 ± 0.17(b)		
		5.810 ± 0.69	2.350 ± 0.30		
Phenobarbital (8)	3.256 ± 0.41*(a)	2.748 ± 0.49(a)	1.243 ± 0.27(a)	0.508 ± 0.20*(a)	10.565 ± 1.00(b)
		1.771 ± 0.24(b)	0.644 ± 0.11(b)		
		4.519 ± 0.70	1.887 ± 0.35		

Values are expressed as μmoles leucine/g cell protein.
* Significantly different from control at 0.05 level by Student t-test.
(a) [^{14}C] labeled protein.
(b) [^{3}H] labeled protein.
Rate constants of degradation, and half-lives calculated from the initial and final cell protein content (Fig. 1A) corrected for the secretion during 60 min of incubation (Fig. 1B) are: Normal cell: Kd/hr = 0.121 (t 1/2 = 5.73 hr). PB-treated cells Kd/hr = 0.053 (t 1/2 = 13.08 hr).

DISCUSSION

Isolated hepatocytes are now being widely used to study various aspects of liver metabolism; in particular they possess definite advantages in so far they avoid the wastage of labeled molecules in the entire organism and the interference of external homeostatic controls other than changes in concentration of precursor or effector molecules. The double-isotope technique as originally described by Arias *et al.* [23] in whole animals has its main field of application in the study of turnover-time in

long-duration experiments but reutilization of the label may become a critical limitation to the method and relative data, rather than absolute values, are mostly obtained [24]. With isolated cells used in short-term experiments the danger of reutilization of the label is largely overcome by the presence in the medium of a balanced amino acid mixture at five times their plasma concentration which has the following consequences: (a) protein synthesis is stimulated due to the double function of amino acids, as energy-providing molecules [25, 26] as well as building blocks for the growing peptide chain. (b) The rate of protein synthesis is independent of the rate of proteolysis which can be relevant only when amino acids are present in limited amounts. (c) The label released from degraded protein molecules is largely diluted by unlabeled amino acid and its reutilization is practically abolished. (d) Proteolysis by lysosomal enzymes, effecting all classes of cellular proteins is largely inhibited, thus allowing the estimation of the degradation of the more unstable proteins [27]. The use of one and the same amino acid labeled with either [^{14}C] or [^{3}H] in subsequent phases of the experiment offers additional possibilities and gives a more comprehensive idea of protein metabolism without the need of suppressing one or more steps of the pathways (e.g. by appropriate inhibitors) in order to study the others. Rates obtained with isolated hepatocytes from normal rats can be compared with those obtained either *in vivo* or in perfused liver on the basis of the acceptable assumptions that protein constitutes 22% of the liver mass and leucine represent 10.3% by weight of total protein and 11.4% of albumin; then, the synthesis of total protein is 3.29 mg/g of liver/hr, the secretion of total protein is 1.63 mg and that of albumin 0.59 mg/g of liver/hr. These values are similar to those reported in the literature [28, 29].

When the double-isotope technique is applied to hepatocytes isolated from PB-treated rats it is clear that reduction of catabolism is quantitatively more important than increase in protein synthesis in determining the balance in protein content. Since protein content in PB-treated rats increases when related to DNA, our results of protein synthesis, which are expressed as specific activities of liver proteins, could become a little higher than normal if DNA was taken as a reference basis. When the new reference basis is applied to the measure of protein catabolism, the parameters used for calculation of the latter value may change a little but there is always a significant lowering of protein degradation in PB-treated rat liver cells.

The importance of the regulation of protein catabolism in growth processes has already been shown with regenerating livers [1] and with some types of tumours [3, 4]; the present observations seem to reinforce the general validity of this mechanism. Isolation and characterization of the various proteins would hopefully reveal if under particular circumstances, this general mechanism operates selectively on the single molecular species.

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