

ANALYTICAL STUDY OF MICROSOMES AND ISOLATED SUBCELLULAR MEMBRANES FROM RAT LIVER

I. Biochemical Methods

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ABSTRACT

The series introduced by this paper reports the results of a detailed analysis of the microsomal fraction from rat liver by density gradient centrifugation. The biochemical methods used throughout this work for the determination of monoamine oxidase, NADH cytochrome *c* reductase, NADPH cytochrome *c* reductase, cytochrome oxidase, catalase, aminopyrine demethylase, cytochromes *b₅* and P 450, glucuronyltransferase, galactosyltransferase, esterase, alkaline and acid phosphatases, 5'-nucleotidase, glucose 6-phosphatase, alkaline phosphodiesterase I, *N*-acetyl- β -glucosaminidase, β -glucuronidase, nucleoside diphosphatase, aldolase, fumarase, glutamine synthetase, protein, phospholipid, cholesterol, and RNA are described and justified when necessary.

INTRODUCTION

Fractionating the cytoplasmic extract from rat liver by differential centrifugation yields two main particulate fractions: the large granules or mitochondrial fraction and the small granules or microsomal fraction. The analysis of the large granules by differential sedimentation in stabilizing gradients (10) and by isopycnic equilibration in various media (10, 11) has resolved the mitochondrial fraction into three main populations of particles: mitochondria, lysosomes, and peroxisomes. Analysis of the small granules by a similar approach forms the object of this series of papers.

The biochemical study of microsomes started in

1938 when Claude isolated by differential centrifugation a subcellular fraction composed of very small particles (16) later called microsomes (17). Three characteristic structural entities: rough vesicles, smooth vesicles, and ribosomes, were described in this fraction by Palade and Siekevitz (54). These authors have concluded that the major microsomal components¹ arise from endo-

¹In accordance with previous conventions (23) we consistently designate the morphological entities (mitochondria and other organelles) by the word *component* and the biochemical entities (protein, phospholipid, RNA, cholesterol, enzymes) by the word *constituent*.

plasmic reticulum (ER)² by a pinching-off process during homogenization. Henceforth, a great deal of effort has been devoted to the separation of these three entities with the hope of recognizing specific functions associated with each of them.

Microsomes were subfractionated by differential centrifugation in homogeneous medium (15, 42, 47, 49, 54), through a stabilizing density gradient (35), or through sucrose layers (15, 21) and by isopycnic equilibration in density gradients (22, 27, 60). A parallel way was followed by authors preparing submicrosomal fractions by the use of detergents, proteolytic enzymes, mechanical disruption, or organic solvent (19, 20, 31, 36, 43, 55). Much valuable information has been obtained in this manner but, except for the function of ribosomes in protein synthesis, no unambiguous relationship between structure and biochemical characters has been established.

In fact, the microsomal fraction is rather more complex than it was first believed. Elements of the Golgi complex, fragments of the plasma membranes, secretory vesicles, mitochondrial components, endocytic vacuoles, and other subcellular entities may contribute to the composition of the microsomes as well as vesicles deriving from ER. Further, as a biochemical counterpart of that expected cytological complexity, numerous enzymatic activities have been found in the microsomal fraction (69). The intracellular localization of these enzymes has yet to be assessed accurately.

In principle, the cytological localization of enzymatic and chemical constituents can be elucidated by two different biochemical approaches (23, 24). The preparative approach aims at purifying each microsomal component and at determining the constitution of the purified preparations. This approach, which has been extensively applied, suffers from some limitations particularly severe when one is dealing with microsomes. (a) One depends on the complete purification of different microsomal components, an almost inaccessible goal, in view of the great dispersion of densities and sizes (upon which the separation by centrifu-

gation processes relies) within the various populations of microsomal particles (3, 54). (b) The purity of the preparations is evaluated by morphological criteria, and these are often of limited help when the topographical ordering of the cytoplasm has been suppressed by homogenization. For instance, a given smooth microsomal vesicle cannot be unambiguously traced back to its origin in the smooth ER, in the Golgi complex, in the plasma membrane, or in other types of cytomembranes. (c) One will attempt to purify only those membranous entities which have been previously identified by electron microscopy. However, biochemically different membranes do not necessarily appear different under the electron microscope. Biochemical as well as morphological criteria should thus be taken into account in individualizing the various types of cytomembranes.

For these reasons we decided to follow a truly analytical approach in our study on rat liver microsomes. These particles were separated by various density gradient centrifugation procedures into 15 or more subfractions, without attempt at immediate purification of some microsomal component, but instead on the sole basis of physical properties of the particles: equilibrium density or sedimentation coefficient. The subfractions were then assayed for a large number of enzymes and chemical constituents in order to establish quantitatively the distribution patterns of these components. Our hope was to distinguish various groups of constituents on the basis of a common particular behavior in one or several types of subfractionation experiments. The results will be reported in subsequent papers dealing with: (a) the fractionation of rat liver homogenates with the goal of preparing a representative microsomal fraction (2); (b) the analysis of rat liver microsomes by isopycnic equilibration and by differential sedimentation (9); (c) the influence of various treatments on the behavior of microsomal constituents in isopycnic equilibration and differential sedimentation; and (d) the action of digitonin and other agents on several types of subcellular membranes. Some results were presented in a condensed form (1, 3, 4, 8, 25, 70). The morphometric analysis of the microsomal fraction was reported previously (74).

The present paper describes the biochemical methods applied throughout this work. Some modifications or improvements of published techniques are explained and justified.

² *Abbreviations used in this paper:* BAL (British anti-lewisite), 2,3-dimercapto-1-propanol; BSA, bovine serum albumin; dimethyl-POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene; DOC, deoxycholate; ER, endoplasmic reticulum; 4-MeU, 4-methylumbelliferone; PPO, 2,5-diphenyloxazole; UDPGA, uridine diphosphoglucuronic acid; UDP-Gal, uridine diphosphogalactose.

MATERIALS AND METHODS

Unless otherwise stated, enzymatic assays were performed on subcellular fractions suitably diluted with ice-cold tridistilled water, and results are expressed in international units (micromoles of substrate utilized or of product formed per minute). Blanks were normally run in the presence of all constituents of the assay medium with omission of substrate. When significant, substrate blanks were also measured and subtracted.

Spectrophotometric Methods

Time-course determinations were carried out with a Guilford 2000 spectrophotometer (Guilford Instrument Laboratories, Oberlin, Ohio), equipped with cuvette positioner. The cuvette compartment temperature was controlled by a circulating water bath. The spectrophotometer was connected to a linear/log Varicord model 43 recorder (Photovolt Corp., New York) or to a Varian G 1010 (Varian Associates Instrument Div., Palo Alto, Calif.). The linear relationship between absorbance and output signal of the spectrophotometer allows the scale to be expanded by voltage suppression without loss of sensitivity. For this purpose, an automatic voltage suppressor was inserted between the output of the spectrophotometer and the input of the recorder (Fig. 1). The voltage suppressor consists of a regulated source of DC voltage, a fraction of which is automatically opposed to the output voltage of the photometer whenever the input of the recorder is overloaded. Reactions obeying zero-order kinetics are recorded by selecting the linear mode, with 0.2 absorbance full scale. Thanks to the automatic suppression of voltage, several cuvettes can be run simultaneously at that sensitivity, even if they differ from one another by more than 0.6 unit optical density.

NADH and NADPH cytochrome *c* reductases were measured by following the reduction of cytochrome *c* at 550 nm (39, 40). After dilution, the fractions were kept 15 min at 0°C. Then 0.2-ml samples were added to 2.7 ml of medium equilibrated at 25°C and containing 40 mM K phosphate buffer pH 7.4, 32 mM nicotinamide, 0.25 mM Na cyanide, 50 μ M oxidized cytochrome *c*. The reaction was started by adding 0.1 ml of 2.25 mM NADH or NADPH and followed for 10 min. The extinction coefficient for reduced cytochrome *c* was taken to be 19.6 $\text{cm}^{-1} \text{mM}^{-1}$ (41).

Fumarase was assayed by following the increase in absorbance at 240 nm caused by dehydration of L-malate (57). The fractions were preincubated at 25°C. After 10 min, 0.5-ml samples were added to 2.5 ml of solution containing 60 mM K phosphate buffer pH 7.4 and 24 mM Na L-malate. The absorbance was recorded for about 8 min. The extinc-

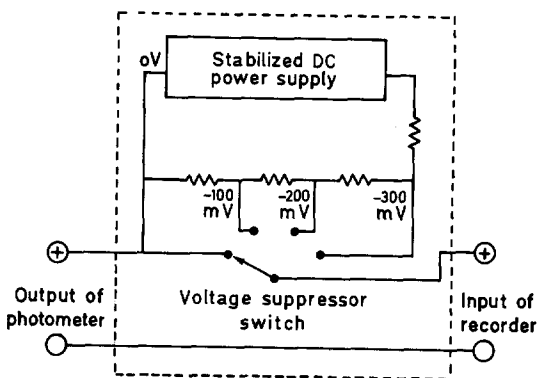


FIGURE 1 Scale expansion by automatic voltage suppression. The recorder is adjusted to 100 mV full scale sensitivity. It is provided with two limit switches, one at each end of the scale, actuated by the pen carriage when it reaches the corresponding limit. These switches control electronically the rotation of the four-position voltage suppressor switch. The setting is stable when none of the limit switches are actuated. A positive overload at the input of the recorder causes the pen carriage to press the 100 mV limit switch and turns the voltage suppressor switch to the right, step by step, until the recorder is balanced again. With a negative overload at the input of the recorder, the 0 mV limit switch is pressed and the voltage suppressor switch is turned to the left in the same manner. Input voltages ranging between 0 and 400 mV are thus recorded with a sensitivity of 100 mV full scale. If necessary, the position of the suppressor switch can be indicated on the chart by a combination of two event markers.

tion coefficient for fumarate was taken to be 2.11 $\text{cm}^{-1} \text{mM}^{-1}$.

Esterase was assayed by following at 420 nm the production of *o*-nitrophenol (extinction coefficient = 3.06 $\text{cm}^{-1} \text{mM}^{-1}$) from *o*-nitrophenyl acetate (13). To 2.7 ml of medium containing 20 mM K phosphate buffer pH 7.4, 1 mM EDTA, and 0.1% Triton X-100, 0.25 ml of fraction was added. After 10 min at 25°C the reaction was started by addition of 0.05 ml of 0.18 M substrate in ice-cold methanol and followed for 10 min. A blank without enzyme was run simultaneously in order to correct for spontaneous substrate hydrolysis.

Cytochrome oxidase was assayed by following the oxidation of cytochrome *c* at 550 nm (5, 18). Since this enzyme obeys first order kinetics, the log mode of the Varicord recorder was selected to plot the time-course of the colog_{10} of reduced cytochrome *c* concentration. It provided a straight line, the slope of which is proportional to the activity (45). The fractions were diluted with an ice-cold solution containing 1 mM EDTA, 1 mM Na bicarbonate, and 0.01% Triton X-100. The reaction was started by addition of 0.1-ml enzyme to 3 ml of 44 μ M cyto-

chrome c reduced up to 80–90% by Na dithionite, 30 mM K phosphate buffer pH 7.4, and 1 mM EDTA. It was run at 25°C for 4 min against a reference cuvette containing oxidized cytochrome c and other reagents at the same concentration. Cooperstein and Lazarow units were given by the slope expressed in $\Delta \log_{10} \text{min}^{-1}$ multiplied by 0.031 (18).

The double beam Cary model 15 spectrophotometer (Cary Instruments, Monrovia, Calif.) was used to determine cytochromes b_5 and P 450. The fractions were diluted with 0.15 M K phosphate buffer pH 7.5. For assaying cytochrome b_5 , the difference spectrum between the oxidized preparation and a sample reduced by 63 μM NADH (through the action of the associated reductase) was recorded between 400 and 430 nm. The concentration of cytochrome b_5 was calculated from the difference: $\Delta\epsilon_{424-405 \text{ nm}} = 160 \text{ cm}^{-1} \text{ mM}^{-1}$ (44). Cytochrome P 450 was determined on preparations reduced with a few grains of $\text{Na}_2\text{S}_2\text{O}_4$, from the difference spectrum between a CO-treated sample and the non-treated preparation. The concentration of P 450 was calculated from the difference: $\Delta\epsilon_{450-490 \text{ nm}} = 91 \text{ cm}^{-1} \text{ mM}^{-1}$ (52).

Automated Determinations

Automated methods were applied for the determination of protein, acid phosphatase, 5'-nucleotidase, and catalase. Since the actual time of incubation in the Technicon assembly cannot be known with accuracy, a manual determination of enzyme activity was also performed on the cytoplasmic extract and the nuclear fraction (2), to convert the absorbances recorded into enzyme activity units. The manual assay was performed with reagents at the

same concentration as in the incubation coil of the automatic apparatus. Technicon Auto-Analyzer modules (Technicon Instruments Corp., Tarrytown, N. Y.) were used. The photometer was a Guilford 300 microsample instrument. A flow cell of 25- μl volume and 1.5-cm light path was devised for high resolution. The absorbance was registered by a Varian G 14 A-1 recorder fed through an automatic voltage suppressor (see Fig. 1) to expand the scale when necessary, with a constant sensitivity of 0.4 absorbance unit full scale. Manifolds were designed for optimum sensitivity and accuracy. In particular, it was necessary to suppress the jerky motion of the liquid emerging from the incubation coils. The air bubbles were removed (C_1 of Figs. 2 and 3) and the solution was partially pumped through the peristaltic pump, as the remaining portion went off to waste. The solution was then joined with a stream of Folin reagent (protein determination) or NH_4 molybdate (phosphatase assays) and segmented again by air.

For protein determination (Fig. 2), Na deoxycholate (DOC) was added to regularize the flow rate and solubilize the membranous proteins. NaOH concentration in the Na_2CO_3 -NaOH reagent was adjusted to neutralize about 90% of the phosphoric acid of the Folin-Ciocalteu reagent. The color development was then less sensitive to the concentration of alkali (46).

For total acid phosphatase determination (Fig. 3), the reaction medium contained 50 mM Na β -glycerophosphate, 50 mM Na acetate-acetic acid buffer pH 5, and 0.07% Triton X-100 (26, 72). Assay for 5'-nucleotidase was performed in a medium containing 2 mM Na AMP, 50 mM Tris-HCl buffer pH 7.5, 8 mM MgCl_2 , 100 mM KCl, and 0.15% Triton

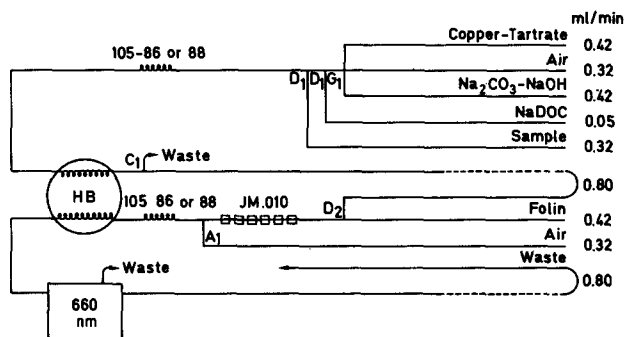


FIGURE 2 Automated protein determination. JM.010, jet mixer, alternate segments of Tygon tubing of 0.010 and 0.0625 inch ID. HB, heating bath, 37°C, twice 40 feet coil, 1.6 mm ID. C_1 is utilized as debubbler. Other symbols refer to Technicon catalog (Technicon Instruments Corp., Tarrytown, N. Y.). Copper-tartrate: solution containing 1.6 mM CuSO_4 and 2.83 mM Na-K tartrate. Na_2CO_3 -NaOH: solution containing 755 mM Na_2CO_3 and 340 mM NaOH. NaDOC: 2%. Folin: freshly prepared eightfold aqueous dilution of Folin-Ciocalteu reagent, titrated vs. NaOH 0.5 N (10 ml Folin = 5 ml NaOH). Washing fluid: water. Standard: 0.005% bovine serum albumin (BSA) in water. Sampling rate: 20 samples per h. Colorimetry at 660 nm.

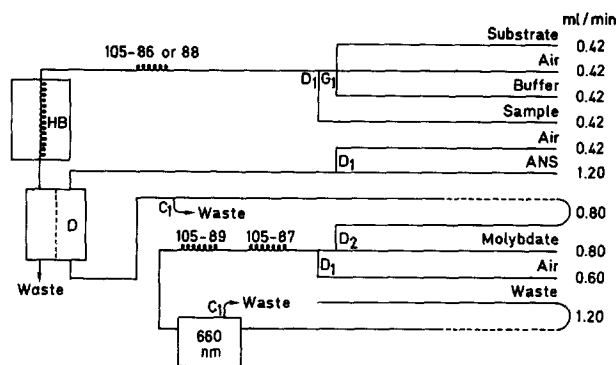


FIGURE 3 Automated phosphatase assay. HB, heating bath 37°C, 20-m Teflon tubing 16 Std (Penntube Plastics Co., Clifton Heights, Pa.), coiled around a plastic cylinder, diameter 20 cm. The bubble pattern is very regular and can be observed at any time all along the coil. Resistance to breakage, flexibility, and reduction of tailing are other advantages of the Teflon coil. C₁ is utilized as debubbler. Other symbols refer to Technicon catalog. Acid phosphatase: Substrate: 0.15 M Na β-glycerophosphate brought to pH 5 with HCl and containing 0.2% Triton X-100. Buffer: 0.15 M Na acetate-acetic acid pH 5. Washing fluid: 0.01% Triton X-100, 1 mM EDTA, 1 mM Na bicarbonate. Samples are diluted in the same fluid. 5'-Nucleotidase: Substrate: 6 mM Na adenosine 5'-monophosphate. Buffer: 0.15 M Tris-HCl pH 7.5 containing 24 mM MgCl₂, 300 mM KCl, and 0.5% Triton X-100. Washing fluid: 5 mM Tris-HCl pH 7.5 with 5 mM MgCl₂ and 0.01% Triton X-100. Samples are diluted in the same fluid. ANS: freshly prepared tenfold aqueous dilution of 0.2% solution of aminonaphthol sulfonic acid in 1.44 M NaHSO₃-Na₂SO₃, supplemented with Na dodecyl sulfate to give a final concentration of 0.4%. Molybdate: fourfold dilution of 0.02 M NH₄ molybdate in 5 N H₂SO₄ supplemented with Na dodecyl sulfate to give a final concentration of 0.4%. Standard: 1 mM KH₂PO₄ in 0.01 N H₂SO₄. Sampling rate: 20 samples per h. Colorimetry at 660 nm.

X-100 (29). After about 25-min incubation, inorganic phosphate was partially removed by dialysis and measured colorimetrically (32).

Catalase was assayed as described by Leighton et al. (45). One unit of activity is defined as the amount of enzyme destroying 90% of the substrate in 1 min in a volume of 50 ml under the conditions of assay.

Other Enzymatic Determinations

Glucose 6-phosphatase was assayed in 1-ml final volume: enzyme was incubated 30 min at 37°C, with 40 mM K glucose 6-phosphate, 20 mM histidine-HCl buffer pH 6.5, and 1 mM EDTA (26). The reaction was stopped by addition of 5 ml of 8% TCA. Inorganic phosphate was determined on 1-ml aliquots of filtrate in 5-ml final volume (32).

Nucleoside diphosphatase activity was assayed by adding 0.1 ml of fraction diluted in 0.1% Na DOC, to 0.5 ml of a mixture containing 5 mM Na IDP, 4 mM MgCl₂, and 50 mM Tris-HCl buffer pH 7.5 (30). After 20-min incubation at 37°C, 4.4 ml of 0.14 M ice-cold perchloric acid and 1 ml of 40 mM NH₄ molybdate in 4 N H₂SO₄ were added. The phosphomolybdate complex was extracted in 6 ml isobutanol and measured on aliquots of 3 ml after reduction by SnCl₂ (73).

Glucuronyltransferase was assayed with 4-methyl-

umbelliferone (4-MeU), or with bilirubin as acceptor. In its principle the method followed in the first case is identical to that developed independently by Arias (6). The 4-MeU conjugated by enzymic action was freed from the nonconjugated fluorochrome by extraction with ethyl ether and hydrolyzed thereafter by added β-glucuronidase; the 4-MeU released from the conjugated product was then measured by fluorometry. The enzyme (0.1 ml) diluted in 0.1% Brij-35 (Sigma Chemical Co., St. Louis, Mo.) was incubated for 20 min at 37°C in 0.4-ml final volume containing 1 mM uridine diphosphoglucuronic acid (UDPGA), 1 mM 4-MeU, 0.1 M Tris-HCl buffer pH 7.3, 0.075% bovine serum albumin (BSA), 2 mM Na ATP, and 8 mM EDTA. The reaction was stopped by adding 3 ml of 6.7% TCA. After centrifugation an aliquot was extracted repeatedly by ethyl ether to remove TCA and unconjugated 4-MeU, heated at 80°C to drive out ether, and incubated with β-glucuronidase for 30 min at 37°C in 0.2 M Na acetate-acetic acid buffer pH 5. Finally, 2 vol of 0.5 M Na carbonate-bicarbonate buffer were added and fluorescence was measured at 450 nm (excitation wavelength 375 nm) with a double monochromator spectrofluorometer ZFM 4 (Carl Zeiss, Oberkochen, Germany). UDPGA was omitted in the blanks. Standardization was made by replacing UDPGA with 0.1 mM 4-MeU-glucuronide.

Assay of bilirubin-UDP-glucuronyltransferase was adapted from Van Roy and Heirwegh (71). The conjugated bilirubin produced was determined from the absorbance at 530 nm of the azo-pigments formed by coupling with the diazonium salt of ethyl anthranilate. Bilirubin (5 mg) was dissolved in 0.5 ml of 0.05 N NaOH, diluted with 9.5 ml BSA (24 mg/ml), and used immediately. The enzyme (0.1 ml), diluted in Brij-35, was incubated for 30 min at 37°C in 0.5-ml final volume containing 0.34 mM bilirubin, 4 mM UDPGA, 10 mM Mg acetate, 0.9% BSA, and 100 mM Tris-HCl buffer pH 7.4. The enzymatic reaction was stopped with 1 ml of 0.5 M glycine-HCl pH 2.65, and incubation was pursued for 30 min at 37°C after addition of 1 ml chromogen. This reagent was prepared in the dark just before use by dispersing 0.1 ml ethyl anthranilate in 10 ml of 0.15 N HCl; 0.3 ml of NaNO₂ (5 mg/ml) was then added and the mixture was stirred for 5 min; finally 0.1 ml of NH₄ sulfamate was added. Development of color reaction was stopped by adding 0.5 ml of ascorbic acid (80 mg/ml). The azo-pigments were extracted by 5 ml methyl *n*-propylketone:*n*-butyl acetate (17:3). After centrifugation, the upper layer was separated and its absorbance at 530 nm was read, with extraction solvent as reference. UDPGA was omitted in the blanks. The extinction coefficient of the azo-pigment derivatives of conjugated bilirubin was taken to be 44.4 cm⁻¹ mM⁻¹.

Galactosyltransferase was assayed by measuring the transfer of labeled galactose from uridine diphosphogalactose (UDP-Gal) to ovalbumin. In 0.1-ml final volume, 0.1 M cacodylate-HCl buffer pH 6.2, 1.25 mM UDP-[U-¹⁴C]Gal diluted to a specific activity of 0.4 mCi/mmol with cold UDP-Gal, 10 mM ATP, 10 mM MnCl₂, 30 mM β-mercaptoethanol, 5% ovalbumin, 0.2% Triton X-100 were incubated 60 min at 37°C with 20-μl enzyme. A blank was run simultaneously, in which enzyme was replaced by water. Incubation was ended by addition of 0.2 ml of 8% TCA. After centrifugation the supernate was discarded and the pellet was washed three times with 8% TCA. The dried pellet was dissolved in 0.8 ml 1 N NaOH by heating 20 min at 80°C. After neutralization by 1 ml acetic acid, radioactivity was determined on 1.5-ml aliquots diluted with 10 ml of scintillation mixture containing in 1 liter: 2.67 g 2,5-diphenyloxazole (PPO), 80 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl-POPOP), 667 ml toluol, 333 ml Triton X-100. Alternatively, the reaction was stopped by pipetting 0.05 ml of the incubated mixture on strips (3 × 2 cm) of Whatman no. 31 ET chromatography paper. The strips were washed in TCA and ethanol as described by Reimann et al. (59), and then transferred to liquid scintillation vials containing 10 ml of scintillation mixture (4 g PPO, 120 mg dimethyl-POPOP, 1 liter toluol).

Monoamine oxidase was assayed by measuring the amount of ammonia produced by oxidative deamination of tyramine (7). The following components in a final volume of 1 ml were incubated with enzyme in penicillin flasks closed with a rubber cap: 3 mM substrate, 1 mM EDTA, 80 mM K phosphate buffer pH 7.6. After 16 h at 30°C in a shaking incubator (Gallenkamp, London, England) the reaction was stopped by 1 ml of saturated K carbonate. Ammonia was trapped by a film of 1 N H₂SO₄ on glass rods fixed through the rubber stoppers and protruding inside the flasks disposed in a diffusion unit (Arthur H. Thomas Co., Philadelphia, Pa.) (65). After 1 h of rotation, the glass rods were rinsed with about 7 ml of water and the ammonia was estimated by the method of Russell (61) in a final volume of 10 ml.

Demethylating activity was assayed by measuring the amount of formaldehyde produced by oxidative demethylation of aminopyrine (53). Nicotinamide was omitted because of its inhibitory effect (63). The reaction was started by addition of 0.3 ml of enzyme to 2 ml of medium containing 50 mM Tris-HCl buffer pH 7.5, 5 mM aminopyrine, 5 mM MgCl₂, and a NADPH generating system including 8 mM Na isocitrate, 0.5 mM NADP, 0.01 mM MnCl₂, and 10 μl isocitrate dehydrogenase. The rate of NADP reduction was 0.2 μmol min⁻¹ at room temperature. Due to the rather low activities in some fractions, incubation at 30°C was prolonged 2 h although a loss of activity occurred. The linear relationship between the formaldehyde produced and the amount of microsomes incubated was maintained under such conditions. The reaction was stopped by adding 0.25 ml of saturated Ba(OH)₂ and 0.25 ml of ZnSO₄ solution of same titer. After centrifugation the formaldehyde was measured on 2-ml portions by adding 2 ml of the Nash reagent (50). The color was developed for 30 min at 37°C and read at 420 nm.

Aldolase was assayed by measuring the dinitrophenylhydrazine derivatives of triose phosphates produced from Na D-fructose 1,6-diphosphate (68). Fractions were incubated for 30 min at 37°C with 5 mM substrate, 56 mM hydrazine sulfate, and 40 mM Tris-HCl buffer pH 8.6, in a final volume of 1.25 ml. After addition of 1 ml 10% TCA the tubes were centrifuged and 0.5-ml portions of supernate were treated with 0.5 ml of 0.75 N NaOH for 10 min. 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was then added and the tubes were kept for 10 min at 37°C. Finally, 0.75 N NaOH was added up to 5-ml final volume and the absorbance was read at 540 nm after developing the color for 20 min. Absorbance readings were converted to molar units by an empirical factor based on alkali-labile phosphate determination.

Glutamine synthetase was assayed by measuring the γ-glutamyl hydroxamate produced from gluta-

mate and hydroxylamine in presence of ATP (67). In 1.5-ml final volume, enzyme was incubated with 80 mM Tris-HCl buffer pH 7.2, 50 mM freshly neutralized hydroxylamine-HCl, 40 mM K glutamate, 13.5 mM Na ATP, 20 mM MgCl₂, and 0.5 mM 2,3-dimercapto-1-propanol (BAL). The reaction was stopped by adding 2 ml 8% TCA. After centrifugation, 2 ml of supernate were added to 1 ml 20% FeCl₃ in 0.5 N HCl. Absorbance was read at 500 nm. A calibration curve was prepared with γ -glutamyl hydroxamic acid.

Alkaline phosphatase, alkaline phosphodiesterase I, and *N*-acetyl- β -glucosaminidase were estimated by measuring at 400 nm the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate, *p*-nitrophenyl-thymidine 5'-phosphate, and *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside, respectively.

Alkaline phosphatase was incubated 10 min at 37°C in a volume of 1 ml containing 5 mM substrate, 5 mM Mg acetate, 70 mM KCl, 5 mM glycine-KOH pH 9 (28). The reaction was stopped by adding 3 ml of 0.1 N NaOH. The tubes were immediately cooled at 4°C and centrifuged.

For alkaline phosphodiesterase I, incubation was carried out for 5–15 min at 25°C in 0.5-ml final volume, containing 1.5 mM substrate, 0.1 M glycine-NaOH buffer at pH 9.6, and 2 mM Zn acetate. The reaction was stopped by adding 1 ml 0.1 N NaOH.

N-acetyl- β -glucosaminidase was incubated for 10 min at 37°C, in a total volume of 0.5 ml containing 6 mM substrate, 100 mM Na citrate buffered at pH 4.5 with HCl, and 0.1% Triton X-100 (66). The reaction was stopped by adding 0.5 ml of cold absolute ethanol and the mixture centrifuged. To 0.5 ml of the supernate were added 2.5 ml of 0.5 M NaHCO₃ pH 10.7.

β -Glucuronidase was assayed by measuring at 555 nm the liberation of phenolphthalein from phenolphthalein glucuronide (34). Incubation was carried out for 30–60 min at 37°C in a volume of 1 ml containing 1.25 mM substrate, 50 mM Na acetate buffer pH 5.2, 0.25 M sucrose, and 0.1% Triton X-100. The reaction was stopped by adding 3 ml of a solution at pH 10.7 containing 133 mM glycine, 83 mM Na₂CO₃, and 67 mM NaCl and the mixture was clarified by centrifuging 30 min at 2,000 rpm.

Chemical Constituents

RNA was assayed by the orcinol method for pentoses (64). Since the presence of sucrose interferes with the colorimetric reaction, the first precipitate was washed three times with 10% TCA and twice with ethanol. For extraction of RNA, the sediment was treated 20 min at 90°C with 5 ml 5% TCA. The color was read at 660 nm.

Phospholipids were extracted in methanol:chloroform (2:1) and washed near 0°C with an equal

volume of 0.1 N HCl (12). After centrifugation, the lower phase was separated and evaporated to dryness under low pressure and at a temperature that did not exceed 40°C. 0.2 ml chloroform was used to dissolve the extract. Aliquots of 10 and 20 μ l were mineralized with 1 ml 10 N H₂SO₄ and clarified with hydrogen peroxide. Phosphate was measured by the procedure of Marinetti et al. (48), after hydrolysis of pyrophosphate at 100°C.

Total cholesterol was determined either by the colorimetric reaction of Liebermann-Burchard or by gas chromatography. In both cases, the samples were first submitted to saponification at 100°C for 30 min by 15% aqueous KOH, to which an equivalent volume of ethanol was added for a second similar treatment in the boiling water bath. Cholesterol was then extracted in three successive steps with petroleum ether (bp 40°–60°). The extracts were dehydrated by Na₂SO₄, filtered, and evaporated to dryness.

In the colorimetric assay, the dry residues were dissolved in acetone and treated by an alcoholic solution of digitonin for 12–20 h. The insoluble cholesterol-digitonin complex was collected either by centrifugation or by filtration and washed successively three times by a mixture of ethanol:acetone:water (3:2:1), three times by a mixture of acetone:ethyl ether (1:1), and lastly three times by ethyl ether. After thorough evaporation of the solvents the precipitate was dissolved in 1 ml of warm glacial acetic acid and 1.5 ml of the Liebermann-Burchard reagent (acetic anhydride:H₂SO₄, 20:1) was added. The mixture was kept free from light for 30 min at 25°C and absorbance was read at 640 nm in a Zeiss spectrophotometer. A calibration curve was made with a solution of pure cholesterol in acetone:ethanol (1:1).

For gas chromatography determinations, stigmasterol was chosen as internal standard. A quantity of stigmasterol, more or less equivalent to the expected amount of cholesterol, was first added to the sample. The dry petroleum ether residues were treated for 2 h at room temperature with 0.05–0.20 ml of a mixture of dry pyridine:hexamethyldisilane:trimethylchlorosilane (4:6:3) and evaporated to dryness. Residues were dissolved in small volumes of ethyl acetate and analyzed in a 5450 G gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) with dual flame ionization detectors. The unit was equipped with glass columns (6 feet \times $\frac{1}{8}$ inch) packed with 1.5% silicone gum rubber OV-17 on high performance chromosorb W-AW-DMCS 80/100 (Hewlett-Packard Co.). Column temperature was 265°C.

Materials

UDP-[U-¹⁴C] Gal was purchased from The Radiochemical Centre, Amersham, England. Nicotinamide,

Na dithionite, Na β -glycerophosphate, aminopyrine, K glutamate, and digitonin were products from E. Merck AG, Darmstadt, Germany. 4-MeU, phenolphthalein glucuronide, and *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside were obtained from Koch-Light Laboratories Ltd, Colnbrook, England. B grade *p*-nitrophenyl-thymidine 5'-phosphate was a product from Calbiochem, San Diego, Calif. Isocitrate dehydrogenase contained 20 U/ml and was purchased from Boehringer and Soehne, Mannheim, Germany; Na DOC from Hopkins and Williams, Ltd., Chadwell Heath, England; Triton X-100 from Rhom and Haas, Co., Philadelphia, Pa.; BSA from Poviet Producten, Amsterdam, Holland. All other chemicals of analytical grade were purchased

from Sigma Chemical Co., St. Louis, Mo. Horse heart cytochrome *c* was of grade VI and K glucose 6-phosphate of grade III.

RESULTS AND DISCUSSION

The kinetics of all enzymes studied was examined in detail, to find optimum conditions of assay and in particular to determine the range within which the measured velocity constants were proportional to the enzyme concentration. Most methods were used as previously published by us or by others, or with minor adaptations. Some, however, were thoroughly modified and call for comment.

Glucuronyltransferase

The fluorescent compound 4-MeU was selected as acceptor because it provided enough sensitivity to measure the enzyme in microsomal subfractions. Glucuronyltransferase has been found to be latent in fresh preparations of microsomes and strongly activated by Triton X-100 (75). The concentration of the detergent was critical, for the activation curve went through a narrow maximum beyond which Triton X-100 appeared to inhibit the enzyme (Fig. 4). It was preferred to suppress the latency by using Brij-35 which had no inhibitory effect over a wide range of concentration and induced a higher level of activity. In this respect Brij-35 was also found slightly more efficacious than digitonin. Besides, we observed

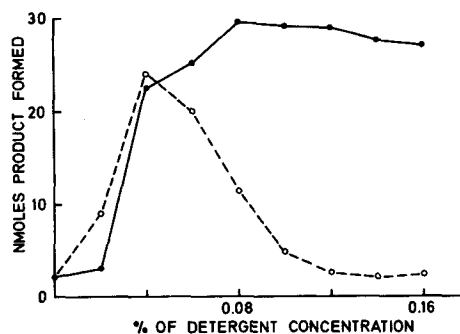


FIGURE 4 Activation of glucuronyltransferase (4-MeU) by Triton X-100 (○) or Brij-35 (●). Assays were carried out as described under Materials and Methods with microsomes from 0.4 mg liver (40 μ g protein/ml) in presence of 0.25 M sucrose.

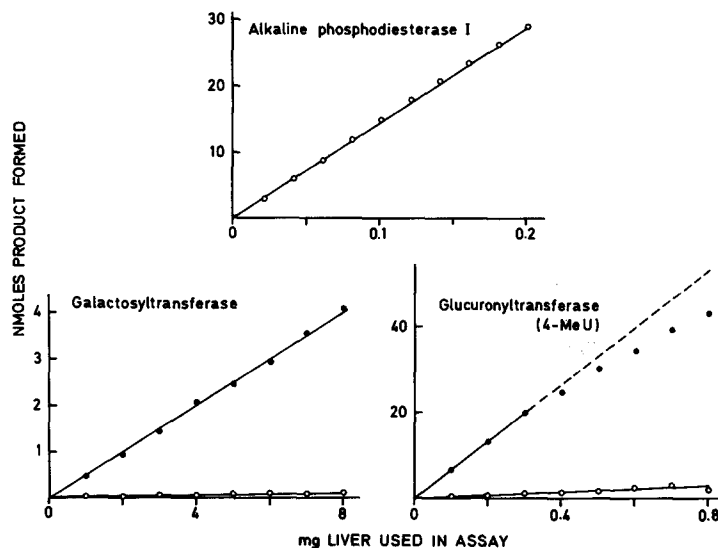


FIGURE 5 Calibration curve of alkaline phosphodiesterase I, galactosyltransferase, and glucuronyltransferase (4-MeU). Assays were carried out as described under Materials and Methods with different amounts of the same microsomal preparation and in presence (●) or in absence (○) of detergent. For galactosyltransferase and glucuronyltransferase the incubation mixture was made 0.25 M in sucrose.

that EDTA and ATP increased the reaction rate whereas $MgCl_2$ was strongly inhibitory. The assay mixture was thus supplemented with EDTA and ATP, which acted probably by protecting UDPGA against hydrolysis by pyrophosphatases (51, 56). Under these conditions the time-course of 4-MeU conjugation is linear for 20 min and the amount of conjugated fluorochrome is proportional, up to 20 nmol, to enzyme concentration (Fig. 5). Beyond, the rate of reaction is markedly decreased. When bilirubin was used as acceptor, the reaction was carried out in presence of Mg^{2+} , since this cation is known to stimulate strongly the bilirubin-UDP-glucuronyltransferase activity of rat liver (38, 71).

Galactosyltransferase

No transfer of galactose to *N*-acetylglucosamine was observed when the method of Fleischer et al. (33) was applied to rat liver microsomes. Chromatographic analysis of the incubated medium showed that the galactose moiety of UDP-Gal appeared to a large extent as galactose 1-phosphate and as free galactose, whether or not *N*-acetylglucosamine was present. Addition of 10 mM ATP suppressed almost completely the degradation of UDP-Gal and revealed the galactosyltransferase activity. The method is, however, somewhat tedious, for the *N*-acetylglucosamine has to be separated from UDP-Gal by adsorption of the latter on Dowex 2X8 (Dow Chemical USA, Membrane Systems Div., Midland, Mich.). Consequently, ovalbumin (62) was preferred as acceptor in routine work. Fig. 5 shows that the transfer of galactose to ovalbumin is proportional to the concentration of enzyme. It is seen further that the enzyme is essentially latent; the activity was increased here more than 30-fold by Triton X-100. The latency is much less pronounced with *N*-acetylglucosamine as acceptor; only a threefold increase in activity was then induced by the detergent.

Alkaline Phosphodiesterase I

The method of Brightwell and Tappel (14) gave inconsistent results, partially due to a rapid inactivation of the enzyme at 37° or 25°C. In agreement with Razzell (58), it was found that the enzyme is very sensitive to EDTA. In some cases a complete inhibition was obtained at 1 μM

TABLE I
Inhibition of Alkaline Phosphodiesterase I by EDTA and Reactivation by Various Salts

Concentration of EDTA	Salt added 5 min after EDTA	Percentage of activity of controls
μM		
0	None	100
1	None	16
1	10 μM $ZnCl_2$	100
5	None	undetectable
5	50 μM $ZnCl_2$	60
5	50 μM $CoCl_2$	30
5	50 μM $NiSO_4$	5
5	50 μM $MgCl_2$, $FeCl_3$, $BaCl_2$, $MnCl_2$, or $BeSO_4$	<5

Microsomes were incubated at 25°C in 1.5 mM *p*-nitrophenyl-thymidine 5'-phosphate buffered at pH 9.6 by 0.1 M glycine-NaOH. Absorbance was measured at 400 nm.

concentration of the chelating agent. The results of a systematic investigation point to Zn^{2+} as activator of the liver enzyme. As shown by Table I, $ZnCl_2$ and, to a lesser extent, $CoCl_2$ reactivated EDTA-inhibited alkaline phosphodiesterase I of rat liver microsomes. 1 mM $ZnCl_2$ enhanced two- to threefold the activity of untreated microsomes. This cation has been shown to activate phosphodiesterase from vegetal source (37). Other salts ($HgCl_2$, $CdCl_2$, $NiSO_4$, $AlCl_3$, Li_2SO_4 , $SrCl_2$, $Co[NO_3]_2$, $MgCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$) were inactive or inhibitory. Besides, alkaline phosphodiesterase was not activated by Triton X-100, Na DOC, or digitonin.

Automated Assays

Recording of acid phosphatase, 5'-nucleotidase and protein determinations are shown in Fig. 6 which presents the corresponding calibration curves. Glucose 6-phosphatase was assayed manually because the active samples interfered with the subsequent ones in the automated assay. Interference was due to deposition of active material on the walls of the coils by agglutination. As a consequence the return to base line was very slow. All attempts to avoid this trouble by adding detergents have been unsuccessful, for glucose 6-phosphatase was strongly inhibited at the concentra-

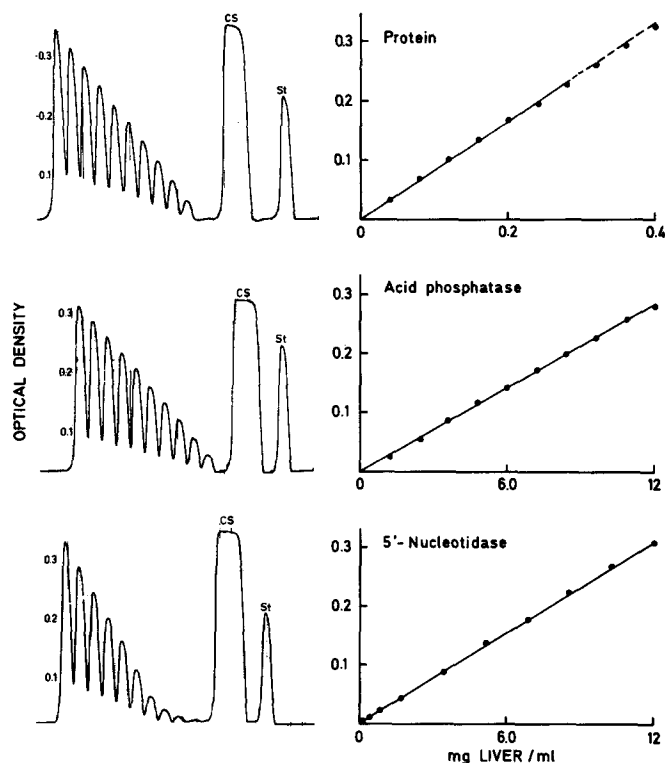


FIGURE 6 Calibration of automated assays. On the left-hand side, experimental tracings obtained from the right to the left with increasing concentrations of cytoplasmic extract. St, standards: serum albumin ($50 \mu\text{g}/\text{ml}$) or phosphate (1mM). CS, maximum concentration of cytoplasmic extract (last peak) sampled continuously. On the right-hand side, optical densities at the peaks, expressed from the base line, are plotted vs. the concentration of cytoplasmic extract.

tions required to "solubilize" the membranes. Such problems were not encountered with the two other phosphatases assayed either because the particles do not agglutinate at pH 7.5 (5'-nucleotidase) or because the enzyme is released in soluble form by Triton X-100 (acid phosphatase).

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REFERENCES

1. AMAR-COSTESECC, A. 1973. Etude analytique des membranes subcellulaires par centrifugation en gradient de densité. Application à la fraction microsomiale de foie de rat. *Spectra 2000*. 4:137.
2. AMAR-COSTESECC, A., H. BEAUFAY, J. BERTHET, M. WIBO, D. THINES-SEMPOUX, E. FEYTMANS, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* 61:201.

3. AMAR-COSTESECC, A., H. BEAUFAY, E. FEYTMANS, D. THINES-SEMPoux, and J. BERTHET. 1969. Subfractionation of rat liver microsomes. In *Microsomes and Drug Oxidations*. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, editors. Academic Press, Inc., New York, 41.
4. AMAR-COSTESECC, A., H. BEAUFAY, D. THINES-SEMPoux, and J. BERTHET. 1968. Heterogeneity of the membranes of the microsomal fraction. *Excerpta Med. Int. Congr. Ser.* 166:38.
5. APPELMANS, F., R. WATTIAUX, and C. DE DUVE. 1955. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem. J.* 59:438.
6. ARIAS, I. M. 1962. Chronic unconjugated hyperbilirubinemia without overt signs of hemolysis in adolescents and adults. *J. Clin. Invest.* 41: 2233.
7. BAUDHUIN, P., H. BEAUFAY, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, P. JACQUES, and C. DE DUVE. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* 92:179.
8. BEAUFAY, H. 1971. Le fractionnement des membranes cellulaires. *Bull. Acad. Med. Belg.* 7e série. 11:315.
9. BEAUFAY, H., A. AMAR-COSTESECC, D. THINES-SEMPoux, M. WIBO, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* 61:213.
10. BEAUFAY, H., D. S. BENDALL, P. BAUDHUIN, R. WATTIAUX, and C. DE DUVE. 1959. Tissue fractionation studies. 13. Analysis of mitochondrial fractions from rat liver by density-gradient centrifuging. *Biochem. J.* 73:628.
11. BEAUFAY, H., P. JACQUES, P. BAUDHUIN, O. Z. SELLINGER, J. BERTHET, and C. DE DUVE. 1964. Tissue fractionation studies. 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* 92:184.
12. BLIGH, E. G., and W. J. DYER. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911.
13. BOWERS, W. E., J. T. FINKENSTAEDT, and C. DE DUVE. 1967. Lysosomes in lymphoid tissue. I. The measurement of hydrolytic activities in whole homogenates. *J. Cell Biol.* 32:325.
14. BRIGHTWELL, R., and A. L. TAPPEL. 1968. Subcellular distributions and properties of rat liver phosphodiesterases. *Arch. Biochem. Biophys.* 124:325.
15. CHAUVEAU, J., Y. MOULE, C. ROULLER, and J. SCHNEEBELI. 1962. Isolation of smooth vesicles and free ribosomes from rat liver microsomes. *J. Cell Biol.* 12:17.
16. CLAUDE, A. 1938. A fraction from normal chick embryo similar to the tumor producing fraction of chicken tumor I. *Proc. Soc. Exp. Biol. Med.* 39:398.
17. CLAUDE, A. 1943. The constitution of protoplasm. *Science (Wash. D. C.)*. 97:451.
18. COOPERSTEIN, S. J., and A. LAZAROW. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* 189:665.
19. DALLMAN, P. R., G. DALLNER, A. BERGSTRAND, and L. ERNSTER. 1969. Heterogeneous distribution of enzymes in submicrosomal membrane fragments. *J. Cell Biol.* 41:357.
20. DALLNER, G. 1963. Studies on the structural and enzymic organization of the membranous elements of liver microsomes. *Acta Pathol. Microbiol. Scand. Suppl.* 166:1.
21. DALLNER, G., P. SIEKEVITZ, and G. E. PALADE. 1966. Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *J. Cell Biol.* 30:73.
22. DECLOITRE, F., and J. CHAUVEAU. 1968. Fractionnement des microsomes de foie de rat. I. Caractères biochimiques et activités enzymatiques des subfractions obtenues sur gradient de densité. *Bull. Soc. Chim. Biol.* 50:491.
23. DE DUVE, C. 1964. Principles of tissue fractionation. *J. Theor. Biol.* 6:33.
24. DE DUVE, C. 1967. General principles. In *Enzyme Cytology*. D. B. Roodyn, editor. Academic Press Inc. Ltd., London, 1.
25. DE DUVE, C. 1971. Tissue fractionation. Past and present. *J. Cell Biol.* 50:20 D.
26. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 60:604.
27. EL-AASER, A. A., E. REID, E. KLUCIS, P. ALEXANDER, J. T. LETT, and J. SMITH. 1966. Resolution of the components in the microsomal fraction of liver in the B-IV zonal centrifuge. *Natl. Cancer Inst. Monogr.* 21:323.
28. EMMELot, P., and C. J. Bos. 1966. Studies on plasma membranes. II. K⁺-dependent p-nitrophenyl phosphatase activity of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta.* 121:375.

29. EMMELOF, P., C. J. BOS, E. L. BENEDETTI, and PH. RÜMKE. 1964. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta.* **90**:126.
30. ERNSTER, L., and L. C. JONES. 1962. A study of the nucleoside tri- and diphosphate activities of rat liver microsomes. *J. Cell Biol.* **15**:563.
31. ERNSTER, L., P. SIEKEVITZ, and G. E. PALADE. 1962. Enzyme-structure relationships in the endoplasmic reticulum of rat liver. A morphological and biochemical study. *J. Cell Biol.* **15**:541.
32. FISKE, C. H., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375.
33. FLEISCHER, B., S. FLEISCHER, and H. OZAWA. 1969. Isolation and characterization of Golgi membranes from bovine liver. *J. Cell Biol.* **43**:59.
34. GIANETTO, R., and C. DE DUVE. 1955. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β -glucuronidase and cathepsin by rat-liver particles. *Biochem. J.* **59**:433.
35. GLAUMANN, H., and G. DALLNER. 1970. Sub-fractionation of smooth microsomes from rat liver. *J. Cell Biol.* **47**:34.
36. HALLINAN, T., and H. N. MUNRO. 1965. A rapid method for preparing granular and agranular endoplasmic reticulum and free ribosomes from rat liver. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **50**:93.
37. HARVEY, C., L. MALSMAN, and A. L. NUSSEBAUM. 1967. A phosphodiesterase from the carrot. *Biochemistry.* **6**:3689.
38. HEIRWEGH, K. P. M., V. VAN DE VIJVER, and J. FEVERY. 1972. Assay and properties of digitonin-activated bilirubin uridine diphosphate glucuronyltransferase from rat liver. *Biochem. J.* **129**:605.
39. HOGEBOOM, G. H. 1949. Cytochemical studies of mammalian tissues. II. The distribution of diphosphopyridine nucleotide-cytochrome *c* reductase in rat liver fractions. *J. Biol. Chem.* **177**:847.
40. HOGEBOOM, G. H., and W. C. SCHNEIDER. 1950. Cytochemical studies of mammalian tissues. III. Isocitric dehydrogenase and triphosphopyridine nucleotide-cytochrome *c* reductase of mouse liver. *J. Biol. Chem.* **186**:417.
41. HORECKER, B. L., and L. A. HEPPEL. 1949. The reduction of cytochrome *c* by xanthine oxidase. *J. Biol. Chem.* **178**:683.
42. IMAI, Y., A. ITO, and R. SATO. 1966. Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem.* **60**:417.
43. ITO, A., and R. SATO. 1969. Proteolytic microdissection of smooth-surfaced vesicles of liver microsomes. *J. Cell Biol.* **40**:179.
44. KLINGENBERG, M. 1958. Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **75**:376.
45. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. FOWLER, and C. DE DUVE. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical, and morphological properties of fractions. *J. Cell Biol.* **37**:482.
46. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.
47. MANGANIELLO, V. C., and A. H. PHILLIPS. 1965. The relationship between ribosomes and the endoplasmic reticulum during protein synthesis. *J. Biol. Chem.* **240**:3951.
48. MARINETTI, G. V., M. ALBRECHT, T. FORD, and E. STOTZ. 1959. Analysis of human plasma phosphatides by paper chromatography. *Biochim. Biophys. Acta.* **36**:4.
49. MOULE, Y., C. ROULLER, and J. CHAUVEAU. 1960. A biochemical and morphological study of rat liver microsomes. *J. Biophys. Biochem. Cytol.* **7**:547.
50. NASH, T. 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416.
51. OGAWA, H., M. SAWADA, and M. KAWADA. 1966. Purification and properties of a pyrophosphatase from rat liver microsomes capable of catalysing the hydrolysis of UDP-glucuronic acid. *J. Biochem. (Tokyo).* **59**:126.
52. OMURA, T., and R. SATO. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370.
53. ÖRRENIUS, S. 1965. On the mechanism of drug hydroxylation in rat liver microsomes. *J. Cell Biol.* **26**:713.
54. PALADE, G. E., and P. SIEKEVITZ. 1956. Pancreatic microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2**:671.
55. PASCAUD, A., P. B. AULIAC, J.-C. EHRHART, and M. PASCAUD. 1970. Protéines constitutives des membranes microsomiques de la cellule hépatique du rat. *Biochim. Biophys. Acta.* **219**:339.
56. POGELL, B. M., and L. F. LELOIR. 1961. Nucleotide activation of liver microsomal glucuronidation. *J. Biol. Chem.* **236**:293.
57. RACKER, E. 1950. Spectrophotometric measure-

- ments of the enzymatic formation of fumaric and cis-aconitic acids. *Biochim. Biophys. Acta.* 4:211.
58. RAZZEL, W. E. 1968. Polynucleotidase activity of animal and plant tissue phosphodiesterases. *Can. J. Biochem.* 46:1.
 59. REIMANN, E. M., D. A. WALSH, and E. G. KREBS. 1971. Purification and properties of rabbit skeletal muscle adenosine 3',5'-monophosphate-dependent protein kinases. *J. Biol. Chem.* 246:1986.
 60. ROTHSCHILD, J. 1963. The isolation of microsomal membranes. *Biochem. Soc. Symp.* 22:4.
 61. RUSSELL, J. A. 1944. The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. *J. Biol. Chem.* 156:457.
 62. SCHANBACHER, F. L., and K. E. EBNER. 1970. Galactosyltransferase acceptor specificity of the lactose synthetase A protein. *J. Biol. Chem.* 245:5057.
 63. SCHENKMAN, J. B., J. A. BALL, and R. W. ESTABROOK. 1967. On the use of nicotinamide in assays for microsomal mixed-function oxidase activity. *Biochem. Pharmacol.* 16:1071.
 64. SCHNEIDER, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis. *Methods Enzymol.* 3:680.
 65. SELIGSON, D., and K. HIRAHARA. 1957. The measurement of ammonia in whole blood, erythrocytes, and plasma. *J. Lab. Clin. Med.* 49:962.
 66. SELLINGER, O. Z., H. BEAUFAY, P. JACQUES, A. DOYEN, and C. DE DUVE. 1960. Tissue fractionation studies. 15. Intracellular distribution and properties of β -N-acetylglucosaminidase and β -galactosidase in rat liver. *Biochem. J.* 74:450.
 67. SELLINGER, O. Z., and F. DE BALBIAN VERSTER. 1962. Glutamine synthetase of rat cerebral cortex: intracellular distribution and structural latency. *J. Biol. Chem.* 237:2836.
 68. SIBLEY, J. A., and A. L. LEHNINGER. 1949. Determination of aldolase in animal tissues. *J. Biol. Chem.* 177:859.
 69. SIEKEVITZ, P. 1963. Protoplasm: endoplasmic reticulum and microsomes and their properties. *Ann. Rev. Physiol.* 25:15.
 70. THINES-SEMPOUX, D., A. AMAR-COSTESECC, H. BEAUFAY, and J. BERTHET. 1969. The association of cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I with a distinct group of microsomal particles. *J. Cell Biol.* 43:189.
 71. VAN ROY, F. P., and K. P. M. HEIRWEGH. 1968. Determination of bilirubin glucuronide and assay of glucuronyltransferase with bilirubin as acceptor. *Biochem. J.* 107:507.
 72. WATTIAUX, R., and C. DE DUVE. 1956. Tissue fractionation studies. 7. Release of bound hydrolases by means of Triton X-100. *Biochem. J.* 63:606.
 73. WEIL-MALHERBE, H., and R. H. GREEN. 1951. The catalytic effect of molybdate on the hydrolysis of organic phosphate bonds. *Biochem. J.* 49:286.
 74. WIBO, M., A. AMAR-COSTESECC, J. BERTHET, and H. BEAUFAY. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J. Cell Biol.* 51:52.
 75. WINSNES, A. 1969. Studies on the activation in vitro of glucuronyltransferase. *Biochim. Biophys. Acta.* 191:279.