J. Membrane Biol. 22, 329–340 (1975)

Comparison of Some Permeability Properties of Rat Liver Slices, Liver Cells in Suspension, and *in Vivo*-produced Aggregates of Dispersed Liver Cells

G. Kranti Kumar and P.M. Bhargava

Regional Research Laboratory, Hyderabad 500009, India

Received 7 December 1973; revised 26 February 1975

Summary. It has been reported earlier that when rat liver is dispersed to a single cell suspension, the parenchymal cells lose the ability to take up pyrimidine bases but acquire the ability to take up RNAase and macromolecular nucleic acids. It is now shown that these changes are largely reversed on intraperitoneal reaggregation of the parenchymal cells and that, in these respects, the aggregates behave more like the organized tissue than like the dispersed cells.

Qualitative differences have been reported from this laboratory between rat liver slices and liver parenchymal cell suspensions in their ability to take up orotic acid, adenine, uracil, RNAase, RNA and DNA, or to secrete serum albumin or leak out nucleotides (Jacob & Bhargava, 1964, 1965; Shanmugam & Bhargava, 1966, 1969; Bhargava, 1968, 1970; Kumar & Bhargava, 1969, 1972, 1973; Bhargava & Shanmugam, 1970; Kumar, 1971; Hussain & Bhargava, 1974). Dissociation of other tissues from higher organisms to a single cell suspension has also been reported to result in surface changes (*inter alia*) (Barnard, Weiss & Ratcliffe, 1969; Cuatrecasas, 1971*a, b*). In sea-urchin embryos, dispersion to a single cell suspension leads to a decreased permeability to phosphate (Sconzo, Pirrone, Mutolo & Giudice, 1970).

Since aggregates obtained from tissue cell suspensions represent a level of organization between that of the whole tissue and the cell suspensions, we have now studied the uptake of orotic acid, uracil, RNAase and macromolecular RNA and DNA by aggregates obtained on intraperitoneal injection of a suspension of rat liver parenchymal cells. We have found that the aggregates resemble liver slices and not the cells in suspension in regard to the uptake of the above substances.

Materials and Methods

Animals

Albino rats of either sex, of inbred strains obtained from the Haffkine Institute, Bombay, and the National Institute of Nutrition, Hyderabad, were used.

Media

Locke's solution and Krebs-Ringer's phosphate (KRP) buffer were prepared as described by Dawson, Elliott, Elliott and Jones (1959) and Umbreit, Burris and Stauffer (1949), respectively, excepting that Ca^{2+} was omitted in both cases and glucose (15 mM) was added to the KRP buffer.

Radioactive Materials

6-^{14}C-orotic acid, 2-^{14}C-uracil, ^{3}H-uridine (G), ^{32}P-sodium phosphate, and carrier-free ^{131}I-sodium iodide were obtained from the Bhabha Atomic Research Centre, Bombay, India. ^{131}I-labeled bovine pancreatic RNAase was prepared and purified as described earlier (Kumar & Bhargava, 1973). ^{32}P-labeled total *E. coli* RNA was prepared as described by Shanmugam and Bhargava (1969). ^{32}P-labeled *E. coli* DNA was prepared and purified essentially as the RNA, with the modification that the extraction with phenol was done at 4 °C in 0.01 M Tris-HCl buffer containing NaCl (0.1 M), EDTA (0.001 M) and SDS (1.0%), at a final pH of 8.5, following lysis of the bacteria in the above buffer at 60 °C. In the labeled nucleic acid preparations used, all the radioactivity was shown to be in internucleotide linkage.

Tissue Preparations

The liver parenchymal cell suspensions were prepared by the method of Jacob and Bhargava (1962), with the exception that the perfusion with citrate and the dispersion of cells were carried out at 37 °C and that Locke's solution was used for the dispersion instead of sucrose (cf. Pertoft, 1969; apGwynn, Jones, Jones & Kemp, 1970; Suzanger & Dickson, 1970). All the experiments reported here, excepting those on the uptake of uridine, were also repeated with cells prepared by the original method of Jacob and Bhargava (1962) in which both perfusion and dispersion are carried out in the cold; the results obtained were virtually identical with those given here.

Liver slices (0.5 mm thick) were cut free-hand with a razor blade, from the citrate-perfused livers (Jacob & Bhargava, 1962).

The aggregates of the liver parenchymal cells were obtained by injection of the parenchymal cells in suspension (1 ml, $8–10 \times 10^6$ cells) intraperitoneally into animals of the same sex and strain as the donor animals (Laws & Stickland, 1961). The recipient animals were sacrificed 10–12 hr after the injection, and the aggregates—which could be observed as faintly yellow, irregular masses, 1–4 mm in diameter, loosely adhering to intraperitoneal adipose and the mesenteric membrane—were picked out and freed of extraneous tissue by fine-tipped forceps. Using ^{32}P-labeled cells and estimation of DNA and protein, it was shown that at least 75% of the injected cells were recovered in the aggregates.

In any one experiment, the cells in suspension and the slices used directly for the uptake studies were obtained from the same liver or from the same group of pooled livers, while the cells used for forming the aggregates were obtained from a different group of animals. In some experiments, the slices and the cell suspension were also obtained from the liver of the recipient animal in which the aggregates were produced; the uptake values for these

slices and cell suspension were within 5% of the value for the corresponding preparation from the donor animals from which the cell suspension for producing the aggregates in the recipient animal was derived. The results given are of typical experiments.

Incubation

The parenchymal cells in suspension (1.5–5.3 mg protein/ml), the slices (5.5–15.9 mg protein/ml) or the aggregates (6.2–9.2 mg protein/ml), were incubated with the labeled substrate in 3.0 ml (final volume) of KRP buffer containing penicillin (100 IU/ml), in 25-ml Erlenmeyer flasks at 37 °C with shaking (25–30 oscillations/min) in a constant temperature water bath; a separate flask was used for each time point.

Estimation of the Uptake

At the end of the incubation period, the tissue preparation was rapidly washed 6–8 times in the cold, with KRP buffer containing an excess of the unlabeled substrate, until the washings were virtually free of radioactivity; the cells in suspension were washed by centrifugation at $50 \times g$ for 2 min, and the slices and aggregates by decantation. (In experiments on the uptake of RNA and DNA, the last two washes were carried out with the buffer without addition of the unlabeled substrate.) The washed tissue preparation was treated with 5% trichloroacetic acid (TCA) in the cold, and the TCA precipitate was washed once each with cold 5% TCA, ethanol-ether (3:1) and ether; the first TCA supernatant and the TCA washing were pooled. Radioactivity was estimated in the combined TCA supernatants and in the TCA-precipitate dissolved in 0.6 N NaOH, in a Packard Tricarb liquid scintillation spectrometer in Bray's scintillation cocktail (^{14}C and ^{3}H), or in a Tracerlab Gamma-guard spectrometer (^{131}I). In experiments on the uptake of RNA and DNA, the nucleic acids were extracted from the washed tissue preparation as described under preparation of the labeled *E. coli* nucleic acids, and radioactivity was estimated by the Cerenkov radiation method in the Packard liquid scintillation spectrometer. Protein was estimated in the defatted TCA precipitate by the method of Lowry, Rosebrough, Farr and Randall (1951), and RNA and DNA by measuring the $E^{1\,cm}_{260\,nm}$ (an absorbance of 1.0 was taken as equivalent to 40 μg of RNA and 50 μg of DNA). In no case was more than 10% (usually much less) of the substrate taken up.

Results and Discussion

In the case of ^{14}C-orotic acid (Fig. 1), ^{14}C-uracil (Fig. 2) and ^{3}H-uridine (Fig. 3), the uptake was measured in the acid-soluble and the acid-insoluble fractions separately and then totalled; separate values for the two fractions are given in Table 1. In the case of ^{131}I-RNAase (Fig. 4) and *E. coli* ^{32}P-DNA (Fig. 5), the uptake was measured in the acid-insoluble fraction; no acid-soluble radioactivity was found (Kumar, 1971; Kumar & Bhargava, 1973). The uptake of *E. coli* ^{32}P-RNA (Fig. 6) was represented by the label observed in total cellular RNA, and excluded RNA which was taken up but degraded in the cells (Shanmugam & Bhargava, 1969). In all cases, the uptake values given represent the net amount transported.

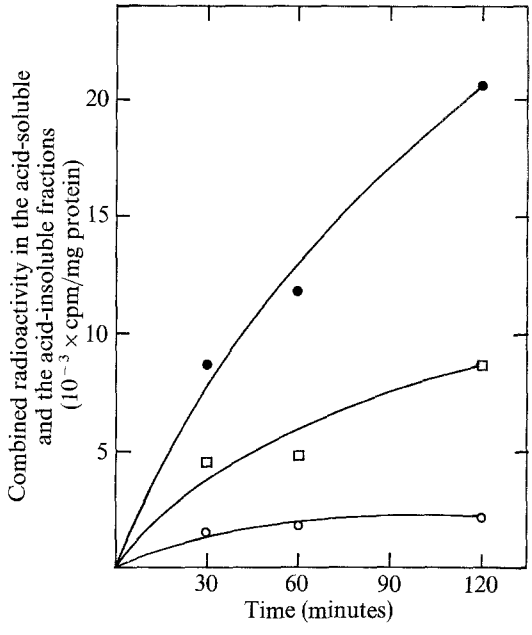

Fig. 1. Uptake of ^{14}C-orotic acid by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with 6-^{14}C-orotic acid [0.068 μmoles (3.69×10^6 cpm)/ml], washed free of external radioactivity, and the uptake in the acid-soluble and the acid-insoluble fractions estimated, as described in the text. The total uptake is expressed as per mg of protein in the acid-insoluble fraction

The amount of orotic acid and uridine transported into the liver cell aggregates at 2 hr was 41 and 97%, respectively, of the amount transported in liver slices (Figs. 1 and 2); the corresponding values for liver cells in suspension were only 10 and 6%, respectively. Table 1 shows that the reduced uptake in the cell suspension was not a consequence of a relatively inefficient incorporation of the precursor into nucleic acids, as whatever was taken up was, in fact, incorporated into RNA more efficiently in the cell suspensions than in the slices or the aggregates.

The uptake of RNAase, DNA and RNA in the aggregates was 49, 35 and 4%, respectively, of that in the cells in suspension (Figs. 4–6); the corresponding values for the slices were 9, 8 and 1%, respectively. The aggregates thus behaved more like the slices than like the cells in suspension in regard to the uptake of these macromolecules.

While the difference between the cells in suspension and the slices in regard to the uptake of the pyrimidine bases and the macromolecules was qualitative, the cells in suspension took up uridine at a rate which was 50% of that obtained in the slices. The aggregates took up uridine

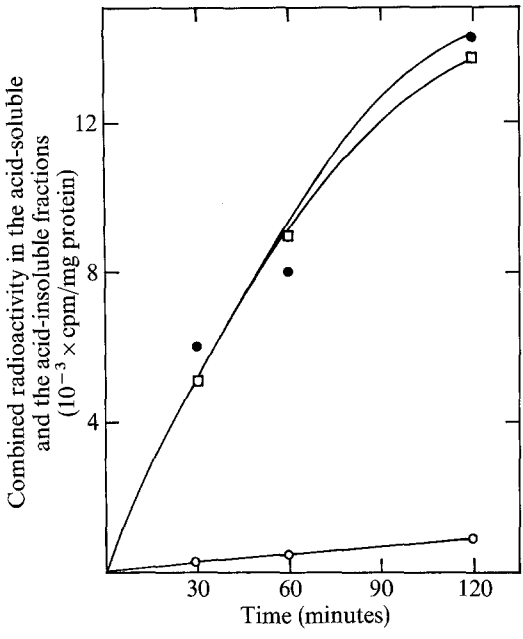

Fig. 2. Uptake of ^{14}C-uracil by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with 2-^{14}C-uracil [0.17 μmoles (3.88×10^6 cpm)/ml] and the total uptake estimated as in Fig. 1

at a rate twice that obtained in the slices and, therefore, again, resembled slices more than the cell suspensions.

The above observations suggest that the behavior of liver parenchymal cells in regard to the uptake of both small and large molecules, depends on whether they exist as single cells or in an organized state as in the slices or the aggregates. However, for this conclusion to be valid it is necessary to examine if the observed differences could not be due to artefacts.

The inability of the cells in suspension to take up orotic acid and uracil (Jacob & Bhargava, 1964, 1965; Bhargava, 1968, 1970) efficiently cannot be a consequence of metabolic death, as cell suspensions prepared by the method used in this investigation, respire; oxidize many of the commonly oxidizable substrates; incorporate amino acids into protein (including albumin); synthesize RNA; synthesize, take up and metabolize lipids (Jungalwala & Dawson, 1970; for other references for the properties listed so far, *see* the introduction, p. 329 and Bhargava, 1968, 1970); can produce interferon in response to viral inducers (P.M. Bhargava & E. DeMaeyer, *unpublished observations*); can transport uridine and incorporate it into RNA at rates comparable to that obtained in the slices

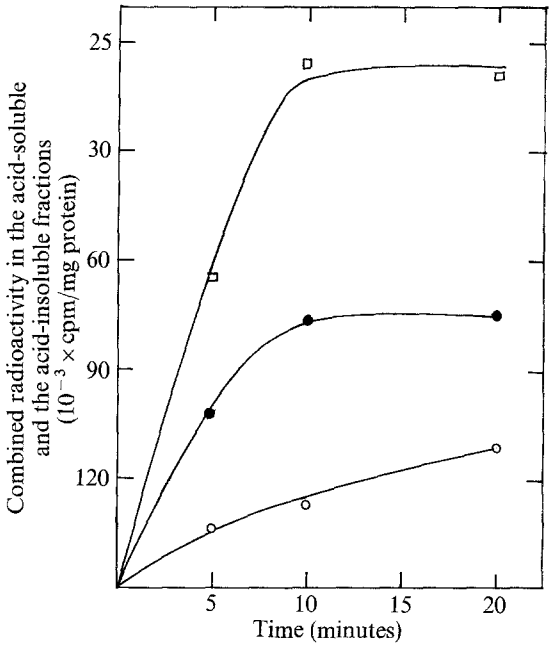

Fig. 3. Uptake of ^{3}H-uridine by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with ^{3}H-uridine (G) [0.0029 μmoles (3.43×10^6 cpm)/ml] and the total uptake estimated as in Fig. 1

(Fig. 3; Table 1); are viable by the criteria of stainability by trypan blue and the ability to transport α-aminoisobutyric acid and cycloleucine against a concentration gradient (Dickson, 1970); and appear largely undamaged in the electron-microscope (apGwynn *et al.*, 1970). Further, the block to transport of the pyrimidine bases is removed on reaggregation of the cells.

The observed uptake of RNA, DNA and RNAase by the cells in suspension cannot be due to adsorption of these materials on the cell surface or to cell debris in the cell suspension, on account of the following reasons: (a) no cell debris was detected in the cell suspensions by microscopic examination under phase contrast; (b) the nucleic acids taken up by the cells were found in one or more of the subcellular fractions such as the cell supernatant, nuclei and mitochondria (Shanmugam & Bhargava, 1966, 1969; Bhargava, 1968, 1970); (c) deoxyribonuclease did not remove any of the labeled DNA taken up by the cells (Kumar, 1971); (d) on incubation of the cell suspensions with RNAase (1 μg/ml), the ribosomal RNA was degraded *within* the cells (Kumar & Bhargava, 1969, 1973).

It may be argued that the observed uptake of pyrimidine bases in

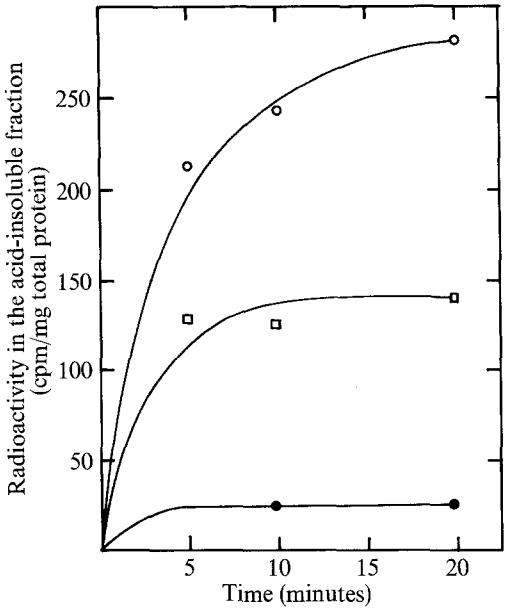

Fig. 4. Uptake of ^{131}I-ribonuclease by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with ^{131}I-ribonuclease [20 μg (11,200 cpm)/ml], washed free of external radioactivity, and the uptake estimated by measurement of radioactivity in the total protein contained in the washed tissue preparation

the aggregates could be due to contamination with other cells such as lymphocytes. Histopathological examination of the aggregates indicated that they consisted almost exclusively of liver parenchymal cells, with less than 2% infiltrating polymorphic and mononuclear lymphocytes; less than 10% of the parenchymal cells appeared autolysed or necrosed. Contaminating cells in the aggregates are therefore unlikely to vitiate the results.

Since parenchymal cells represent 95% of the cellular material in rat liver (Iype, Bhargava & Tasker, 1965), the uptake values obtained for the slices are likely to represent mostly the uptake of the substrate by the parenchymal cells in the slices. Any data on tissue slices must, however, be interpreted with caution as, due to the problem of diffusion in cell multilayers, the slices, cannot be taken implicitly to represent the situation *in vivo*. In this study the comparison of slices with the cells in suspension would appear to be justified as the differences observed were large, verging, in some cases, on the qualitative. Further, if diffusion into the cells, or the cell surface area exposed, were limiting in slices, one would expect the uptake of the pyrimidine bases by the cells in suspension to be greater

Table 1. Comparison of the incorporation of ^{14}C-orotic acid, ^{14}C-uracil and ^{3}H-uridine into acid-insoluble fraction, with their accumulation in the acid-soluble pool, in liver cells in suspension, liver slices and aggregates of the liver cells[a]

Precursor	Time (min)	Uptake (cpm/mg total protein)[b]					
		Cells in suspension		Slices		Aggregates	
		Acid-soluble fraction	Acid-insoluble fraction	Acid-soluble fraction	Acid-insoluble fraction	Acid-soluble fraction	Acid-insoluble fraction
^{14}C-orotic acid	30	1,415 (94.0)	91 (6.0)	8,282 (96.5)	296 (3.5)	4,240 (94.9)	228 (5.1)
	60	1,615 (88.5)	209 (11.5)	11,080 (94.1)	701 (5.9)	4,509 (94.3)	273 (5.7)
	120	1,900 (88.8)	240 (11.2)	19,670 (95.3)	975 (4.7)	7,766 (90.2)	841 (9.8)
^{14}C-uracil	30	118 (44.9)	145 (55.1)	5,496 (91.6)	501 (8.4)	4,503 (98.3)	598 (11.7)
	60	240 (48.5)	255 (51.5)	7,315 (91.3)	700 (8.7)	8,063 (90.0)	900 (10.0)
	120	400 (46.5)	460 (53.5)	12,890 (90.8)	1,301 (9.2)	12,250 (89.1)	1,498 (10.9)
^{3}H-uridine	30	1,050 (39.0)	1,644 (61.0)	7,545 (95.0)	394 (5.0)	12,400 (87.2)	1,828 (12.8)
	60	2,340 (63.0)	1,377 (37.0)	11,630 (94.9)	621 (5.1)	20,970 (87.6)	2,976 (12.4)
	120	3,709 (58.1)	2,676 (41.9)	11,780 (95.6)	539 (4.4)	20,520 (87.6)	2,901 (12.4)

[a] The tissue preparation was incubated with 6-^{14}C-orotic acid [0.068 μmoles (3.69×10^6 cpm)/ml], 2-^{14}C-uracil [0.17 μmoles (3.88×10^6 cpm)/ml] or ^{3}H-uridine (G) [0.0029 μmoles (3.43×10^6 cpm)/ml] and the uptake in the acid-soluble and the acid-insoluble fractions estimated as described in Figs. 1, 2 and 3, respectively.

[b] Figures in parentheses are percentages on the total uptake, i.e. the combined radioactivity in the acid-soluble and the acid-insoluble fractions.

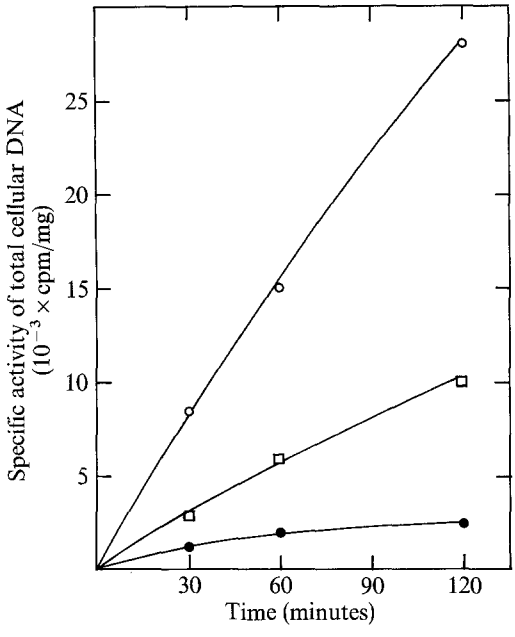

Fig. 5. Uptake of ^{32}P-DNA by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with *E. coli* ^{32}P-DNA [100 μg (56,100 cpm)/ml], washed free of external radioactivity, and the uptake estimated by measurement of radioactivity in the total tissue DNA

than that in the slices, contrary to what was observed. Uptake of DNA in the slices, though low in comparison to the cells in suspensions, was higher than that reported earlier for perfused liver (Ledoux, Gerber, Charles, Remy & Remy-Defraigne, 1967) in which at least some of the above barriers would be lowered. In the case of RNAase, we have already shown that even 1 μg of the enzyme per ml had no effect on cellular RNA in the slices, whereas the RNA in the cell suspensions was degraded with 0.005 μg of the enzyme per ml (Kumar & Bhargava, 1973); such a large difference between the two tissue preparations would be difficult to explain on the basis of an accessibility barrier in the slices.

No kinetic analysis of the uptake properties was made in the study; only gross comparisons were made. These comparisons would appear to be justified as the differences were large and we were dealing with basically the same type of cell in all three cell/tissue preparations.

Our observations are, therefore, strongly indicative of the reversal, on reaggregation, of the changes observed in liver cells on dispersion of the tissue to a single cell suspension. No explanation for these observations is, however, forthcoming from this study. One possibility may be

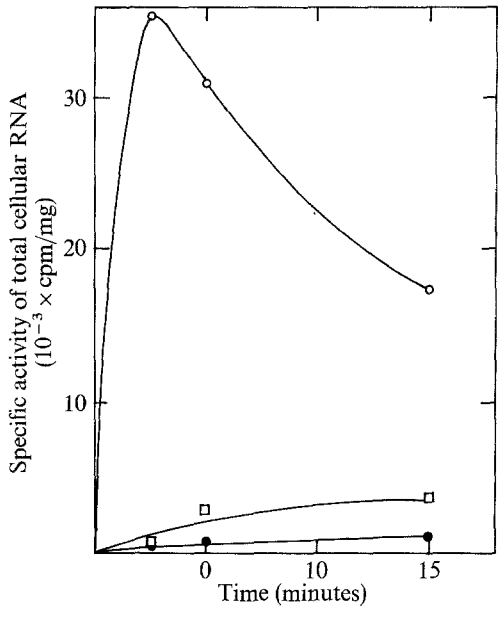

Fig. 6. Uptake of ^{32}P-RNA by liver cells in suspension (0), liver slices (●), and aggregates of the liver cells (□). The tissue preparation was incubated with total *E. coli* ^{32}P-RNA [100 μg (61,900 cpm)/ml], washed free of external radioactivity, and the uptake estimated by measurement of radioactivity in the total tissue RNA

mentioned. Reaggregation *in vitro* is known to be susceptible to actinomycin D, and may require synthesis of some intercellular, protein-containing material (Glaeser, Richmond & Todd, 1968; Overton, 1968; Richmond, Glaeser & Todd, 1968). No aggregates were formed in the present study as well, in 50% of the animals, if actinomycin D (100 μg per rat) was injected along with the cell suspension; the remaining animals had small and scanty aggregates, in contrast to the control group in which all animals had large, well-formed aggregates. The observed permeability differences between the cells in suspension and the tissue slices or the aggregates may, therefore, be related to the presence of intercellular material in the two latter preparations in contrast to the former.

We are grateful to Dr. Prema K. Murthy for the histopathological examination of the aggregates and to the Council of Scientific and Industrial Research, New Delhi, for the award of a Research Fellowship to one of us (G.K.K.).

References

Barnard, P.J., Weiss, L., Ratcliffe, T. 1969. Changes in the surface properties of embryonic chick neural retina cells after dissociation. *Exp. Cell Res.* **54**:293

Bhargava, P.M. 1968. Preparation, properties and uses of single cell suspensions from animal tissues. *Science and Culture (Calcutta)* **34**(Suppl.):105

Bhargava, P.M. 1970. Intercellular control of intracellular metabolic activity. *In:* Control Processes in Multicellular Organisms. G.E.W. Wolstenholme and J. Knight, editors. p. 158. J. and A. Churchill, London

Bhargava, P.M., Shanmugam, G. 1970. Uptake of non-viral nucleic acids by mammalian cells. *Prog. Nucl. Acid Res. Mol. Biol.* **11**:103

Cuatrecasas, P. 1971 *a*. Perturbation of the insulin receptor of isolated fat cells with proteolytic enzymes. Direct measurements of insulin-receptor interactions. *J. Biol. Chem.* **246**:6522

Cuatrecasas, P. 1971 *b*. Unmasking of insulin receptors in fat cells and fat cell membranes. Perturbation of membrane lipids. *J. Biol. Chem.* **246**:6532

Dawson, R.M.C., Elliott, D.C., Elliott, E.H., Jones, K.M. 1959. Data for Biochemical Research. Oxford University Press, London, p. 209

Dickson, J.A. 1970. The uptake of non-metabolizable aminoacids as an index of cell viability *in vitro*. *Exp. Cell Res.* **61**:235

Glaeser, R.M., Richmond, J.E., Todd, P.W. 1968. Histotypic self-organization by trypsin-dissociated and EDTA-dissociated chick embryo cells. *Exp. Cell Res.* **52**:71

apGwynn, I., Jones, B., Jones, B.M., Kemp, R.B. 1970. A modified perfusion technique for preparing isolated liver cells: An ultrastructural study. *Cytobios* **2**:181

Hussain, L.F., Bhargava, P.M. 1974. Liver cells in suspension synthesise but do not secrete serum albumin. *Arch. Biochem. Biophys.* **161**:153

Iype, P.T., Bhargava, P.M., Tasker, A.D. 1965. Some aspects of the chemical and cellular composition of rat liver. *Exp. Cell Res.* **40**:233

Jacob, S.T., Bhargava, P.M. 1962. A new method for the preparation of liver cell suspensions. *Exp. Cell Res.* **27**:453

Jacob, S.T., Bhargava, P.M. 1964. Comparison of the uracil catabolic activity of liver cell suspensions and slices. *Biochim. Biophys. Acta* **91**:650

Jacob, S.T., Bhargava, P.M. 1965. Metabolism of tissue cells in suspension. Synthesis of ribonucleic acid by liver cells in suspension. *Biochem. J.* **95**:568

Jungalwala, F.B., Dawson, R.M.C. 1970. Phospholipid synthesis and exchange in isolated liver cells. *Biochem. J.* **117**:481

Kumar, B.V. 1971. Studies on the metabolism of single cell suspensions. Effect of endogenous and exogenous ribonuclease on liver cells in suspension, and uptake of DNA by the cells. Ph.D. Thesis. Osmania University, Hyderabad, India

Kumar, B.V., Bhargava, P.M. 1969. Effect of ribonuclease on rat liver parenchymal cells in suspension. *Life Sci.* **8**:1103

Kumar, B.V., Bhargava, P.M. 1972. Activation of a ribonuclease on dispersion of rat liver to a single cell suspension and incubation of the cells at 37°. *J. Cell. Physiol.* **80**:175

Kumar, B.V., Bhargava, P.M. 1973. The effect of ribonuclease on rat liver parenchymal cells in suspension. *Biochemie* **55**:161

Laws, J.O., Stickland, L.M. 1961. The adhesion of liver cells. *Exp. Cell Res.* **24**:240

Ledoux, L., Gerber, G.B., Charles, P., Remy, J., Remy-Defraigne, J. 1967. Uptake of labeled DNA by isolated perfused organs. *Experientia* **23**:16

Lowry, O.H., Rosebrough, N.H., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265

Overton, J. 1968. A fibrillar intercellular material in reaggregating embryonic chick cells. *J. Cell Biol.* **39**:101 a

Pertoft, H. 1969. The separation of rat liver cells in colloidal silica-polyethylene glycol gradients. *Exp. Cell Res.* **57**:338

Richmond, J.E., Glaeser, R.M., Todd, P. 1968. Protein synthesis and aggregation of embryonic cells. *Exp. Cell Res.* **52**:43

Sconzo, G., Pirrone, A.M., Mutolo, V., Giudice, G. 1970. Synthesis of ribosomal RNA in disaggregated cells of sea urchin embryos. *Biochim. Biophys. Acta* **199**:441

Shanmugam, G., Bhargava, P.M. 1966. The uptake of homologous ribonucleic acid by rat liver parenchymal cells in suspension. *Biochem. J.* **99**:297

Shanmugam, G., Bhargava, P.M. 1969. Uptake of *Escherichia coli* RNA by rat liver cells in suspension. *Indian J. Biochem.* **6**:64

Suzanger, M., Dickson, J.A. 1970. Biochemical studies on cells isolated from adult rat liver. *Exp. Cell Res.* **63**:353

Umbreit, W.W., Burris, R.H., Stauffer, J.F. 1949. Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, p. 119