The Effect of Actinomycin D on the Synthesis of Ribonucleic Acid and Protein in Rat Liver Parenchymal Cells in Suspension and Liver Slices

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1. Rat liver parenchymal cells in suspension are shown to require a higher concentration of actinomycin D than liver slices for equivalent inhibition of the incorporation of [^{14}C]adenine, [^{14}C]uracil and [^{32}P]phosphate into RNA, and of ^{14}C -labelled amino acids into protein; protein synthesis is much less susceptible to actinomycin D inhibition than RNA synthesis in both the tissue preparations. Possible causes for these differences are discussed. 2. The uptake of [^3H]actinomycin D in the first few minutes was much greater in the cell suspensions than in the tissue slices; that in the next 1–4 hr. was about the same in both the cases. The uptake by both the tissue preparations was at all times proportional to the concentration of the drug within the range 0.5–2.0 $\mu\text{g./ml.}$ 3. In the slices actinomycin D taken up initially was concentrated almost exclusively in the nuclei; with time the concentration of the drug in the mitochondria and the supernatant increased more rapidly than in the nuclei though at no stage did it exceed that in the nuclei. In the cell suspension the largest concentration of the drug taken up initially was found in the supernatant; most of the drug taken up subsequently also stayed in the supernatant. 4. When the drug concentration in the incubation medium was 1 $\mu\text{g./ml.}$, its concentration within the parenchymal cells in suspension and the parenchymal cells in the slices reached 2.2 and 1.6 $\mu\text{g./cm.}^3$ of cellular volume respectively. On average, 7% of the drug was removed from the medium by the cells in suspension and 23% by the cells in the slices; the average ratio of intracellular to extracellular concentration was 2.4 in the former and 2.1 in the latter case.

Though there have been many studies on the effect of actinomycin D on RNA synthesis *in vivo* and in cells in tissue culture, there have been very few reports (Manchester, 1964; Inoue, Hosokawa & Takeda, 1965; Jackson & Sells, 1967) on the effect of this drug on RNA synthesis in mammalian tissue preparations *in vitro*. The present paper describes the effect of a range of concentrations (0.025–75.0 $\mu\text{g./ml.}$) of actinomycin D on RNA synthesis, as judged by the incorporation of several precursors, in rat liver slices and hepatic parenchymal cells in suspension.

The results of some investigations on the transport of [^3H]actinomycin D in the slices and cell suspensions, and on the effect of the drug (0.5–50 $\mu\text{g./ml.}$) on protein synthesis in them, are also reported. Earlier studies on transport of this drug in animal cells *in vitro* were confined to human embryonic cells (Fraccaro, Mannini, Tiepolo & Albertini, 1966) and tumour cells (Harbers, Müller & Backmann, 1963; Goldstein, Hamm & Amrod, 1966) in tissue culture.

MATERIALS AND METHODS

Animals. Adult albino rats (a local inbred strain) of both sexes were used.

Radioactive chemicals. [$8\text{-}^{14}\text{C}$]Adenine and [$2\text{-}^{14}\text{C}$]uracil were obtained from Bhabha Atomic Research Centre (Bombay, India) and The Radiochemical Centre (Amersham, Bucks.). ^{14}C -labelled amino acids and *Chlorella* protein hydrolysate were purchased from the latter, and [^{32}P]phosphate from the former as orthophosphate in 0.9% NaCl buffered with phosphate buffer to give a concentration of 1 mg. of P (approx. 1 mc)/ml. [^3H]Actinomycin D was a gift from Dr E. Harbers and unlabelled actinomycin D a gift from Merck, Sharp and Dohme International, New York, N.Y., U.S.A.

Buffers. Krebs original Ringer phosphate buffer and Krebs–Ringer bicarbonate buffer (Dawson, Elliott, Elliott & Jones, 1959), from which CaCl_2 was omitted and to which glucose was added to a final concentration of 15 mM, were used, the former in experiments with ^{14}C -labelled substrates or [^3H]actinomycin D, and the latter in experiments with [^{32}P]phosphate.

Preparation of liver parenchymal-cell suspensions. The cell suspensions were prepared by the method of Jacob & Bhargava (1962).

Preparation of liver slices. The slices (approx. 500μ thick) were cut freehand with a razor blade from freshly perfused liver. In experiments bearing the same number, the slices and the cell suspensions were derived from the same animal.

Incubations. The tissue preparation (100–160 mg. wet wt. of liver slices, or 2×10^6 – 4×10^6 liver parenchymal cells in suspension, per ml. of the incubation medium) was incubated in the buffer with the labelled precursor in a series of 25 ml. conical flasks (one for each time-point) at 37° in a Gallenkamp constant-temperature water bath with shaking at 100 oscillations/min. Other additions, if any, to the incubation medium were as stated in the legends to the Tables and Figures.

Preparation of dry tissue material. At the end of the incubation, the medium was removed by centrifugation at $200g$ for 5 min.; the sedimented cells or slices were washed twice with the buffer containing an excess of unlabelled actinomycin and then twice with the buffer alone, and dried at 100° after as much of the buffer as possible had been drained off.

Preparation of subcellular fractions. (a) Nuclei. The incubated cells in suspension or slices, washed as above to remove [3H]actinomycin, were further washed once with 0.25 M-sucrose, suspended in 10 ml. of 0.25 M-sucrose and homogenized, first in a Waring Blendor for 2 min. and then in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $600g$ for 15 min. The crude nuclear pellet was purified by dispersal in 6 ml. of 2.2 M-sucrose and centrifuging at $40000g$ for 65 min. in a swinging-bucket (SW39) rotor of a Spinco model L preparative ultracentrifuge (Chauveau, Moulé & Rouiller, 1956); it was resuspended in 0.25 M-sucrose.

(b) Mitochondria. The supernatant obtained during the preparation of crude nuclei was centrifuged at $10000g$ for 30 min. in a Spinco model L ultracentrifuge. The sedimented mitochondrial pellet was resuspended in 0.25 M-sucrose and centrifuged once again as above. The sedimented mitochondria were again suspended in 0.25 M-sucrose.

(c) Supernatant. The incubated cells in suspension or slices were washed as above to remove [3H]actinomycin, suspended in 0.25 M-sucrose and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $105000g$ for 65 min. in a Spinco model L ultracentrifuge and supernatant decanted off.

All the above operations were done in the cold.

Isolation and determination of RNA. Method A. This was used for the isolation of RNA when [^{14}C]adenine or [^{14}C]uracil was used as the precursor. The incubated cells in suspension or slices were treated with an equal volume of cold 20% (w/v) trichloroacetic acid. The precipitate was washed twice each with 5% (w/v) trichloroacetic acid (containing an excess of the unlabelled precursor) and ethanol saturated with sodium acetate. The removal of lipids, extraction of total nucleic acids with 10% (w/v) NaCl and the alkali hydrolysis of total nucleic acids were done as described by Shanmugam & Bhargava (1966).

Method B. This method, used for the isolation of RNA when [^{32}P]phosphate was the precursor, was essentially the same as that described by Scherrer & Darnell (1962) except that the aqueous layer obtained during extraction with phenol was separated by centrifugation at $1500g$ for 30 min. Anionic contaminants were removed by passing the RNA through a Dowex 1 (X8; formate form) column (Petrovic & Petrovic, 1966). Hydrolysis (Shanmugam & Bhargava,

1966) of the RNA eluted from the column, followed by separation of the resulting nucleotides on Dowex 50 (X8) and Dowex 1 (X4) columns (Katz & Comb, 1963), showed that all the radioactivity in the RNA was in internucleotide linkage.

An E_{260} value of 1.0 for the hydrolysed RNA fraction was taken to be equivalent to $33\mu g.$ of unhydrolysed RNA/ml. (Fleck & Munro, 1962).

Isolation and determination of proteins. Proteins were isolated as described by Bhargava & Bhargava (1962) and determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of radioactivity. Solutions of RNA (containing a known amount of the nucleic acid) were plated directly and protein as a slurry in 20% (v/v) ethanol on preweighed aluminium planchets (2 cm.^2); the samples were counted in a Geiger-Müller counter with 6% and 10% efficiency for ^{14}C and ^{32}P respectively. Appropriate correction for self-absorption (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949) was made where necessary.

3H -labelled samples were counted in a Packard Tri-Carb liquid-scintillation counter with 30% efficiency for 3H . For nuclei and mitochondria 0.5 ml. of the suspension containing a known amount of protein was mixed with 10 ml. of the scintillation mixture [0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 1 g. of 2,5-diphenyloxazole, 20 g. of naphthalene and 3 g. of Cab-O-Sil/100 ml. of dioxan]; for the cell supernatant a known volume containing a known amount of protein was extracted exhaustively with ether, and the ether extract was dried and transferred to the vial quantitatively with the aid of 10 ml. of the above scintillation fluid. For measurement of radioactivity in the dried tissue preparation a known amount was mixed in the vial with 10 ml. of the scintillation mixture [0.03 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.5 g. of 2,5-diphenyloxazole/100 ml. of toluene] and 1 ml. of Hyamine; Cab-O-Sil was added until a homogeneous suspension was obtained.

RESULTS AND DISCUSSION

Effect of actinomycin D on the incorporation of labelled adenine, uracil and phosphate into RNA in liver cells in suspension and liver slices

Actinomycin D had no effect on the incorporation of [^{14}C]adenine, [^{14}C]uracil and [^{32}P]phosphate into the RNA of the cell suspensions when the drug was used at concentrations up to $0.5\mu g./\text{ml.}$, at which 40–60% inhibition of the incorporation of these precursors into RNA was obtained in liver slices (Figs. 1 and 2). At concentrations above $0.5\mu g./\text{ml.}$ actinomycin D inhibited the incorporation of these precursors into RNA in the cell suspensions as well; at any concentration the inhibition was less in the cell suspension than in the slices, though this difference became progressively less with increasing concentrations of the drug. The extent of the inhibition was proportional to the logarithm of the concentration of actinomycin D in the range 0.025 – $75\mu g./\text{ml.}$ for slices and 0.5 – $75\mu g./\text{ml.}$ for cell suspensions (Figs. 1 and 2).

Fig. 1. Effect of various concentrations of actinomycin D on the incorporation of $[^{14}\text{C}]$ adenine (a and b) and $[^{14}\text{C}]$ uracil (c and d) into the RNA of liver slices (a and c) and liver parenchymal-cell suspensions (b and d). The tissue preparation was incubated in 3 ml. of the buffer with the stated concentration of actinomycin D and $[^{14}\text{C}]$ adenine $[0.6 \mu\text{C} (0.6 \mu\text{mole})/\text{ml.}]$ or $[^{14}\text{C}]$ uracil $[0.6 \mu\text{C} (0.92 \mu\text{mole})/\text{ml.}]$ at 37° for 240 min. Details of isolation of RNA from the incubated tissue preparation are given in the Materials and Methods section. The percentage inhibition of the incorporation of the precursor into RNA is plotted against the logarithm of the concentration of actinomycin D. A total of 18 experiments were done, in seven of which the tissue slices and the cell suspension were derived from the same animal, in two of which only the slices were studied and in nine of which only the cell suspension was studied. The number given above each experimental point indicates the actual number of experiments in which results were obtained for that particular concentration of actinomycin D, and of which the average is plotted.

Actinomycin D started exhibiting its inhibitory effect on RNA synthesis quite early both in the slices and in the cell suspensions; thus at a concentration of $50 \mu\text{g./ml.}$ the inhibition in both the tissue preparations at 90, 180 and 240 min. ranged between 72 and 85% (Fig. 3).

Fig. 2. Effect of actinomycin D on the incorporation of $[^{32}\text{P}]$ phosphate into the RNA of liver slices (a) and of liver parenchymal-cell suspensions (b). The cells in suspension were incubated in 12 ml. of the buffer and the slices in 6 ml. of the buffer for 180 min. at 37° with the stated concentration of actinomycin D and $[^{32}\text{P}]$ phosphate ($10 \mu\text{C}/\text{ml.}$). Details of the isolation of RNA from the incubated tissue preparations are given in the Materials and Methods section. The percentage inhibition of the incorporation of the precursor into RNA is plotted against the logarithm of the concentration of actinomycin D. The specific radioactivities of RNA in the control (containing no actinomycin) were 15500 and 3985 counts/min./mg. for slices and the cell suspension respectively. Chromatographic analysis (see the text) of the alkali hydrolysate derived from the RNA of liver slices (control sample) containing 4150 counts/min. showed the following distribution of radioactivity (counts/min.) in the various nucleotides: UMP, 1090; GMP, 1270; CMP, 924; AMP, 840; total, 4124; for the RNA of cell suspension (control sample) containing 1500 counts/min. the distribution was: UMP, 536; GMP, 514; CMP, 242; AMP, 235; total, 1527.

In an earlier study Inoue *et al.* (1965) showed that $20 \mu\text{g.}$ of actinomycin D/ml. inhibited by 61% the incorporation of $[^3\text{H}]$ uridine into RNA in liver cell suspensions prepared by the method of Anderson (1953). In rat liver slices obtained from hypophysectomized animals $10 \mu\text{g.}$ of actinomycin D/ml. inhibited by 50–75% the incorporation of $[^3\text{H}]$ orotic acid into the rapidly labelled (45–55s) RNA (Jackson & Sells, 1967). These results are similar to those obtained by us.

Actinomycin D at a concentration of $1 \mu\text{g./ml.}$ or less has been shown to inhibit RNA synthesis by more than 90% in a variety of cells in tissue culture (Harbers & Müller, 1962; Reich, Franklin, Shatkin & Tatum, 1962; Tamaoki & Mueller, 1962; Franklin, 1963; Amos, 1964; Zischka, Langlois & Beard, 1964; Armentrout, Schmickel & Simmons, 1965; Bloom, Goldberg & Green, 1965; Roberts & Newman, 1966). Mammalian cells when grown in

Fig. 3. Effect of actinomycin D on the incorporation of [^{14}C]adenine into the RNA of liver parenchymal cells in suspension (a) and of liver slices (b) at various time-intervals. The tissue preparation was incubated in 3 ml. of the buffer in the absence (O) or in the presence (●) of actinomycin D (50 $\mu\text{g./ml.}$) at 37° for the specified period. The concentration of [^{14}C]adenine in the incubation medium was 0.6 μC (0.6 $\mu\text{mole/ml.}$). Details of the isolation of RNA from the incubated tissue preparations are given in the Materials and Methods section.

tissue culture thus seem to acquire enhanced sensitivity to actinomycin D.

Transport of [^3H]actinomycin D in liver cells in suspension and in liver slices

Slotnick & Sells (1964) have reported that actinomycin D-resistant strains of *Bacillus subtilis* are unable to transport actinomycin D while the protoplasts of these cells are sensitive to this drug. The uptake of the drug by actinomycin D-resistant strains of *Lactobacillus casei* is only about 20% of that by the sensitive strains (Donaldson & Moriarity, 1967). Actinomycin D is apparently not transported in drug-resistant HeLa cells or in the sensitive cells damaged by osmotic shock (Goldstein *et al.* 1966). In HeLa cells that survive the lethal action of actinomycin D, under certain conditions, new mitochondria continue to be formed while nuclear division is inhibited; this

could also be due to the lack of transport of the drug into mitochondria in the surviving cells (Deitch & Godman, 1967). It therefore seemed possible that the lack of inhibition of RNA synthesis in liver cells in suspension by low concentrations of actinomycin D (Figs. 1 and 2) was due to a decrease in the permeability to this drug of the cytoplasmic, nuclear or mitochondrial membranes on dispersion of the liver tissue to a cell suspension. Therefore in a series of experiments the uptake of [^3H]actinomycin D (0.25, 0.5, 1.0 and 2.0 $\mu\text{g./ml.}$) by liver cells in suspension and by liver tissue slices was studied (Table 1); in another series the distribution of [^3H]actinomycin taken up in purified nuclei, mitochondria and the 105 000g supernatant was determined (Table 2).

Uptake of actinomycin D at '0hr.' Table 1 shows that in the slices at all the concentrations of actinomycin D tested the uptake of the drug in the '0hr.' samples was very low (on average about 7% of the peak uptake at 3hr.), whereas in the cell suspension it was quite significant (25–40% of the peak uptake at 2–3hr.), and increased linearly with the concentration of actinomycin D. In the '0hr.' samples 4–5 min. elapsed between the addition of actinomycin D and its removal by centrifugation. It is therefore possible that the uptake of the drug observed at '0hr.', particularly in the cell suspensions, represents an initially rapid uptake; it is, however, referred to subsequently as the '0hr.' uptake. In HeLa cells in tissue culture the uptake of [^3H]actinomycin in the first 10 min. has been reported to be half of the peak uptake at 4hr. (Fraccaro *et al.* 1966).

Uptake of actinomycin D at 1–4hr. The uptake of [^3H]actinomycin D increased (over the '0hr.' uptake) with the time of incubation at all the concentrations of actinomycin D in both the tissue preparations (Table 1); at each time-point the uptake was roughly proportional to the concentration of actinomycin D, as has been reported for Ehrlich ascites-tumour cells (Harbers *et al.* 1963). In the slices the uptake of the drug, at each concentration, continued to rise until 4hr., though the rate of increase fell after 1hr.; in the cell suspensions a plateau was reached at 2–3hr.

Comparison of the uptake of actinomycin D by liver cells in suspension and liver tissue slices. Table 1 shows that the total uptake of actinomycin D (per mg. dry wt. of the tissue preparation) at 0–4hr., as well as the uptake at 1–4hr. expressed as the increase over the '0hr.' uptake, were greater in the cell suspension than in the slices. If, however, the fact that non-cellular material accounts for about half the dry weight of liver slices (Iype, Bhargava & Tasker, 1965) is taken into consideration, and if the uptake by cells other than the parenchymal cells in liver (which represent less

Table 1. *Transport of [³H]actinomycin into liver slices and liver parenchymal-cell suspensions*

The tissue preparation was incubated in 3 ml. of the buffer with the stated concentration of [³H]actinomycin D (10⁸ disintegrations/min./mg.) at 37° for the specified period, washed and dried as described in the Materials and Methods section. The values in parentheses give the increases over the '0 hr.' sample.

Expt. no.	Time of incubation (min.)	Concn. of [³ H]actinomycin (μg./ml.)	Specific radioactivity of dry tissue preparation (counts/min./mg.)							
			Slices				Cell suspension			
			0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0
1	0	6	16	9	27	34	—	140	—	—
	60	24 (18)	82 (66)	183 (174)	—	123 (89)	190	344 (204)	—	—
	120	49 (43)	99 (83)	199 (190)	532 (505)	—	382	540 (400)	—	—
2	0	3	3	5	12	37	64	160	249	—
	180	25 (22)	47 (44)	95 (90)	231 (219)	82 (45)	188 (124)	410 (250)	700 (451)	—
	240	31 (28)	69 (66)	130 (125)	247 (235)	91 (54)	166 (102)	330 (170)	688 (439)	—

Table 2. *Extent of labelling of various subcellular fractions derived from liver slices and liver parenchymal-cell suspensions incubated with [³H]actinomycin D*

The tissue preparation was incubated in 3 ml. of the buffer with the stated concentration of [³H]actinomycin D (10⁸ disintegrations/min./mg.) at 37° for the specified period, washed and the subcellular fractions were isolated as described in the Materials and Methods section. The values in parentheses give the increases over the '0 hr.' sample. In Expt. 4 the radioactivity is expressed for the cell suspension as counts/min. in the total supernatant from cells equivalent to 10 mg. dry wt., and for tissue slices as 2 × counts/min. in the total supernatant from slices equivalent to 10 mg. dry wt.; the observed values for slices were multiplied by 2 to make allowance for the fact that nearly half the dry weight of rat liver is due to non-cellular material (Iype *et al.* 1965).

Expt. no.	Subcellular fraction	Time of incubation (min.)	Concn. of [³ H]actinomycin (μg./ml.)	Radioactivity (counts/min./mg. of protein)					
				Slices			Cell suspension		
				0.25	0.5	2.0	0.25	0.5	2.0
1	Nuclei	0	18	40	236	170	294	1360	—
		120	114 (96)	258 (218)	760 (524)	155 (0)	480 (186)	1750 (390)	—
		120	65 (60)	131 (118)	420 (389)	83 (52)	135 (68)	540 (370)	—
2	Mitochondria	0	5	13	31	31	67	170	—
		120	65 (60)	131 (118)	420 (389)	83 (52)	135 (68)	540 (370)	—
		120	65 (60)	131 (118)	420 (389)	83 (52)	135 (68)	540 (370)	—
3	Supernatant	0	7	3	6	170	535	—	—
		120	72 (65)	192 (189)	700 (694)	755 (585)	1340 (805)	—	—
		120	72 (65)	192 (189)	700 (694)	755 (585)	1340 (805)	—	—
4	Supernatant	0	—	8	16	—	322	1500	—
		180	—	67 (59)	445 (429)	—	1740 (1418)	9550 (8050)	—
		180	—	67 (59)	445 (429)	—	1740 (1418)	9550 (8050)	—
5	Supernatant	0	40	40	130	580	950	3560	—
		180	154 (114)	262 (222)	912 (788)	1200 (620)	2220 (1270)	7530 (3970)	—
		180	154 (114)	262 (222)	912 (788)	1200 (620)	2220 (1270)	7530 (3970)	—

than 10% of the cellular dry weight) is considered negligible, then, though the '0 hr.' uptake of the drug by the parenchymal cells is three- to ten-fold greater in the cell suspensions than in the slices, the uptake in the next 1-4 hr. is about the same in both the cases.

Distribution of [³H]actinomycin D into subcellular fractions from liver cells in suspension and liver slices incubated with the drug

The above experiments do not indicate whether or not actinomycin D is actually transported across the cell membrane in the two tissue preparations

and reaches the sites of transcription (nuclei and mitochondria). It is possible that most or all of the drug taken up by the tissue preparation remained adsorbed on the cell surface, particularly in the cell suspensions, in which the total membrane area having direct access to the drug would be much greater than in the slices.

Experiments described in Table 2 rule out the above possibility. One or more of the subcellular fractions (nuclei, mitochondria, cell supernatant) carried significant radioactivity when either of the tissue preparations was incubated with [³H]-actinomycin D (0.25, 0.5 and 2.0 μg./ml.) for 0-3 hr.; the specific radioactivities (counts/min./mg. of

protein) of the subcellular fractions were about the same as (or higher than) the specific radioactivity (counts/min./mg. dry wt.; Table 1) of the whole tissue preparation from which they were derived. Since liver nuclei, mitochondria and supernatant account for, on average, 15, 23 and 38% of the tissue nitrogen (Schneider & Hogeboom, 1950), this indicates that most of the actinomycin D taken up by the slices or by the cells in suspension was transported into the cell. The radioactivity (wherever significant) in the various subcellular fractions in both the tissue preparations was roughly proportional to the concentration of actinomycin D (Table 2); similar proportionality has been reported for the nuclear uptake of [^{14}C]actinomycin D in Ehrlich ascites-tumour cells (Harbers *et al.* 1963).

These experiments also indicate that in the slices actinomycin D taken up initially, within the first 5 min. (the '0 hr.' uptake), is concentrated almost exclusively in the nuclei, and that with time the drug concentration in the mitochondria and the supernatant increases more rapidly than in the nuclei, though at no stage does it exceed that in the nuclei. One-half to three-quarters of [^3H]actinomycin taken up by the liver tissue *in vivo* has also been shown to be present in the nuclei (Dingman & Sporn, 1965; Weissbach, Redfield, O'Connor & Chirigos, 1966). In ascites-tumour and HeLa cells too, incubated *in vitro* with 1 $\mu\text{g.}$ of [^{14}C]actinomycin D/ml. and 7 $\mu\text{g.}$ of [^3H]actinomycin D/ml. respectively, the drug taken up was found to be almost exclusively localized in the nuclei (Harbers *et al.* 1963; Goldstein *et al.* 1966). On the other hand, in the cell suspensions the drug taken up initially is distributed in all the three subcellular fractions, the largest concentration being in the cell supernatant; most of the actinomycin taken up subsequently also appears to stay in the supernatant, none or very little of it going to nuclei or mitochondria.

Since in Table 2 the results are expressed as counts/min./mg. of protein of the purified subcellular fraction, the values given for nuclei and mitochondria derived from the slices can be directly compared with the values for the corresponding subcellular fraction from the cell suspensions without making any correction for the presence of non-cellular material in the slices. The data of Table 2 therefore show that, at '0 hr.', the ratio of actinomycin D transported into the subcellular fraction derived from the parenchymal cells in suspension to that transported into the corresponding subcellular fraction from the parenchymal cells in the slices was much (perhaps, on average, an order of magnitude) higher for the supernatant than for the nuclei or mitochondria. Further, though the increase on incubation over the '0 hr.' value in the radioactivity transported in the nuclei

and mitochondria derived from the cells in suspension was less than that in the corresponding subcellular fraction from the slices (Table 2, Expt. 1), the increase in the supernatant from the cells in suspension was four to 24 times that observed for the supernatant from the slices (Table 2, Expts. 2-4). It is therefore clear that most (at '0 hr.') or all (at 2 hr.) of the [^3H]actinomycin D taken up by the cells in suspension in excess of that taken up by the parenchymal cells in the slices, at all the concentrations of the drug tested, was present in the cytoplasmic soluble fraction of the parenchymal cells.

It is possible that the '0 hr.' radioactivity in the nuclei and mitochondria represents adsorption on their membrane, and the radioactivity taken up on further incubation at 37° represents transport to the site of RNA synthesis in these subcellular fractions. If this is assumed to be true, the lack of inhibition of RNA synthesis in the cells in suspension by low (0.25 $\mu\text{g./ml.}$) concentrations of actinomycin D could be explained by a decrease in the rate of transport of the drug into the nuclei (and possibly the mitochondria) of these cells, even though the rate of transport from the incubation medium to the cytoplasmic soluble fraction of the cells, and the adsorption of the transported drug on the mitochondrial and nuclear membranes, is increased on dispersion of liver tissue to a cell suspension. Alternatively, the cells in suspension may be preferentially synthesizing RNA species the synthesis of which is intrinsically less susceptible to inhibition by actinomycin D than that of RNA synthesized by the slices. The synthesis of transfer RNA (excluding the turnover of the terminal cytidylylcytidylyladenyllic acid sequence) has been reported to be less susceptible to inhibition by this drug than that of ribosomal RNA or messenger RNA, both *in vivo* (Revel & Hiatt, 1964a; Merits, 1965; Moulé & Landin, 1965) and *in vitro* (Harbers & Müller, 1962; Perry, 1962; Eason, Cline & Smellie, 1963; Franklin, 1963; Gross, Malkin & Moyer, 1964; Perry, Srinivasan & Kelley, 1964). Evidence has also been presented for the presence of other RNA species besides transfer RNA, the synthesis of which is inhibited by actinomycin D to a smaller extent than that of the bulk of the cellular RNA (Paul & Struthers, 1963; Harel, Harel, Boer, Imbenotte & Carpeni, 1964; Klenow & Frederiksen, 1964; Roberts & Newman, 1966).

Comparison of the intracellular and extracellular concentrations of actinomycin D

From the specific radioactivity values in Table 1 (Expt. 1) obtained on incubation of the parenchymal cells in suspension ($3 \times 10^6/\text{ml.}$) or the slices (32.5 mg. dry wt./ml.) with 1 $\mu\text{g.}$ of [^3H]actinomycin

Table 3. *Effect of actinomycin D on the incorporation of ^{14}C -labelled amino acids into the proteins of liver slices and liver parenchymal-cell suspensions*

The tissue preparation was incubated in 3 ml. of the buffer at 37° for the specified period. The concentration of ^{14}C -labelled *Chlorella* protein hydrolysate was $0.33\text{ }\mu\text{C}$ ($0.1\text{ }\mu\text{mole}$ of mixed amino acids)/ml. in Expt. 1 and $0.166\text{ }\mu\text{C}$ ($0.05\text{ }\mu\text{mole}$)/ml. in Expt. 2. The concentrations of [^{14}C]lysine, [^{14}C]proline and [^{14}C]threonine in Expt. 3 were $0.17\text{ }\mu\text{C}$ ($0.025\text{ }\mu\text{mole}$), $0.17\text{ }\mu\text{C}$ ($0.015\text{ }\mu\text{mole}$) and $0.17\text{ }\mu\text{C}$ ($0.028\text{ }\mu\text{mole}$)/ml. respectively. Details of the isolation of protein from the incubated tissue preparations are given in the Materials and Methods section. The values in parentheses give the percentage inhibition of the incorporation.

Expt. no.	Precursor	Tissue preparation	Concn. of actinomycin D ($\mu\text{g./ml.}$)	Specific radioactivity of protein (counts/min./mg.)		
				60 min.	120 min.	240 min.
1	^{14}C -labelled <i>Chlorella</i> protein hydrolysate	Cell suspension	0	350	465	560
			2.5	287 (18)	350 (24)	485 (13)
			12.5	247 (29)	310 (33)	400 (29)
			50	270 (23)	270 (42)	410 (27)
2	^{14}C -labelled <i>Chlorella</i> protein hydrolysate	Slices	0	65	95	101
			2.5	33 (49)	50 (47)	—
			12.5	42 (35)	53 (44)	58 (42)
			50	18 (72)	31 (67)	27 (73)
3	Mixture of [^{14}C]lysine, [^{14}C]proline and [^{14}C]threonine	Slices	0	29	47	72
			0.5	29 (0)	44 (6)	51 (29)
			12.5	21 (27)	30 (36)	38 (47)

D/ml. for 2 hr., the values reported by Iype *et al.* (1965) for the average dry weight ($1.3 \times 10^{-6}\text{ mg./cell}$) and volume ($10.6 \times 10^{-9}\text{ cm.}^3/\text{cell}$) of rat parenchymal cells, and the assumptions that only 50% of the tissue dry weight is contributed by the parenchymal cells and that all the drug taken up by the tissue goes to the parenchymal cells in the slices, the following calculations can be made. (1) The average concentration of actinomycin D in the parenchymal cells in suspension and in the parenchymal cells of the slices was 2.2 and $1.6\text{ }\mu\text{g./cm.}^3$ of cell volume respectively. (2) In the two tissue preparations, 0.018 and $0.014\text{ }\mu\text{g.}$ of the drug was taken up/mg. dry wt. of the parenchymal cells, or 0.023 and $0.018\text{ }\mu\text{g./}10^6$ parenchymal cells respectively. (3) The ratio of intracellular (in parenchymal cells) to extracellular concentration of the drug was 2.4 and 2.1 in the cells in suspension and the slices respectively.

Cultured human embryonic cells (Fraccaro *et al.* 1966) and HeLa cells (Goldstein *et al.* 1966) have been shown to remove 4% (at 3 hr.) and 9% (at 1 hr.) respectively of [^3H]actinomycin D when incubated with 1 and $7\text{ }\mu\text{g.}$ of the drug/ml. Ehrlich ascites-tumour cells have been reported to take up as much as 60% of the drug when incubated with [^{14}C]actinomycin D ($1\text{ }\mu\text{g./ml.}$) for 1 hr. (Harbers *et al.* 1963).

Effect of actinomycin D on protein synthesis in liver cells in suspension and in liver slices

Table 3 shows that protein synthesis in the cell suspensions is less susceptible to inhibition by

actinomycin D than that in the slices. A comparison of the data in Table 3 with those of Figs. 1 and 2 also shows that at any concentration of actinomycin D less inhibition was obtained of protein synthesis than of RNA synthesis in both the tissue preparations; at 2.5, 12.5 and $50\text{ }\mu\text{g.}$ of actinomycin D/ml. the inhibition of protein synthesis was, on average, 18, 30 and 30% in the cell suspensions and 48, 39 and 70% in the slices respectively, whereas that of RNA synthesis was 33, 59 and 81% in the former and 71, 76 and 85% in the latter respectively. This shows that much of the protein synthesized by both the tissue preparations *in vitro* used the messenger that was present before the incubation commenced. This is in line with earlier reports on the stability of liver messenger RNA (Giudice & Novelli, 1963; Revel & Hiatt, 1964b).

Our observations are similar to those of Inoue *et al.* (1965), who observed 20% inhibition of the synthesis of protein by $20\text{ }\mu\text{g.}$ of actinomycin D/ml. in parenchymal cells in suspension prepared by a method similar to the one used by us. Protein synthesis in liver slices has been shown to be virtually unaffected by low concentrations ($1\text{ }\mu\text{g.}$ or less/ml.) of actinomycin D (Nakazato, Hishizawa & Terayama, 1966) that inhibit RNA synthesis up to 90%. In isolated diaphragm high concentrations of the drug ($10\text{ }\mu\text{g./ml.}$) caused only 50–60% inhibition at 6 hr.; the inhibition at earlier time-points was much less (Manchester, 1964); actinomycin D completely inhibits RNA synthesis in this tissue *in vitro* (Manchester, 1964; Eboue-Bonis, Chambaut, Volfin & Clauser, 1963). Actinomycin D ($10\text{ }\mu\text{g./ml.}$) has been shown to have very little effect

on protein synthesis also in ascites-tumour cells (Gvozdev, 1964) and in fertilized eggs (Gross & Cousineau, 1964; Gross *et al.* 1964); in rabbit bone-marrow cells in culture 4 μ g. of the drug/ml. caused only 33% inhibition of protein synthesis in 4 hr. (Armentrout *et al.* 1965), and in L cells 0.2 μ g./ml. (which significantly inhibited RNA synthesis) caused no inhibition of protein synthesis (Reich, Franklin, Shatkin & Tatum, 1961). Thus protein synthesis in mammalian tissues in general *in vitro* seems to be less susceptible to actinomycin D than RNA synthesis, presumably owing to the stability of their messenger.

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