Cytoplasmic Effects of Cortisol in Liver

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1. The time-course of the induction by cortisol of tryptophan pyrrolase in mouse liver was established. 2. The kinetics of labelling of mouse liver polyribosomes and the distribution of radioactivity in the various sizes of polyribosomes were investigated. It is concluded that the major part of the messenger RNA is monocistronic. 3. Cortisol administered to adrenalectomized mice stimulates in parallel the ability of ribosomes and polyribosomes, both free and bound, to incorporate amino acids into protein. 4. This stimulation does not appear to affect in a selective manner particular regions of the gradients of polyribosomes. 5. Administration of cortisol enhances the ability of the denser fraction of the rough microsomes to incorporate labelled amino acids. Adrenalectomy has minor effects on the pool sizes and specific radioactivities of liver amino acids which are counteracted by the hormone. However, these appear insufficient to influence the changes observed. 6. Adrenalectomy causes an increase in the percentage of ribosomes existing as polyribosomes and a fall in the proportion bound to the membrane of the endoplasmic reticulum. After treatment of these animals with cortisol there is a marked increase in the proportion of bound ribosomes. 7. The influence of cortisol on the membranous components of liver cytoplasm is discussed.

Most theories of the regulation of the bacterial operon lay emphasis on control at the level of transcription. However even in micro-organisms various proposals for control at other levels have been made (Epstein & Beckwith, 1968; Cline & Bock, 1966; Cohen, 1966). If, in bacteria, transcription and translation are integrated (Stent, 1964) additional complex forms of regulation must be considered. The mechanisms for control of protein synthesis in eucaryotic organisms are obscure. The physical disengagement of transcription and translation, the attachment of ribosomes to membranes, the dynamic state of proteins and a number of other factors must be borne in mind when comparing micro-organisms with mammalian systems. In the latter, several hormones have been reported to exert specific effects on protein synthesis (for review see Korner, 1967). Our interest in cortisol stemmed from the observation that it induces a number of liver enzymes (Rosen & Nicol, 1964). Examination of the mechanism of induction provides an experimental approach to the problem of control of protein synthesis in high organisms.

One of the most intensively studied of the enzymes whose activity is influenced by glucocorticoids is tryptophan pyrrolase. Administration of cortisol to adrenalectomized rats produces a

fourfold increase in enzyme activity within 3 hr. (Civen & Knox, 1959). Increases in the activity of tryptophan pyrrolase may also be achieved by feeding with tryptophan (Schimke, Sweeney & Berlin, 1965; Knox, 1966). Immunological techniques have been used to demonstrate that the elevation of tryptophan pyrrolase activity is accompanied by a net increase in enzyme protein (Fiegelson & Greengard, 1962). Schimke (1966) showed that, whereas incorporation of radioactive amino acids into total liver protein is increased by 40% within 40 min. after administration of cortisone, incorporation into tryptophan pyrrolase is stimulated by 500%. The interpretation of this observation must take into account the fact that the half-life of tryptophan pyrrolase is 3 hr. whereas the average value for total protein is 72 hr. (Schimke, Sweeney & Berlin, 1964; Knox, 1966).

The apparent selectivity of cortisol in the elevation of the activity of specific enzymes has led many authors to seek an explanation at the level of transcription. Kenney & Kull (1963) demonstrated that the hormone led to a threefold stimulation in the rate of nuclear RNA synthesis in rat liver. Hybridization experiments suggest that cortisol causes the appearance of new species of mRNA† (Drews & Brawerman, 1967), although there is also

† Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA.

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an increase in the synthesis of rRNA and tRNA (Wicks, Greenman & Kenney, 1965). Experiments *in vitro* have suggested that cortisol binds to and activates liver chromatin, thus allowing an increase in mRNA synthesis (Sluysers, 1966). Against this must be weighed the evidence of Dingman & Sporn (1965) and Fiegelson & Fiegelson (1965), which argues against the direct action of cortisol in the nucleus.

Attempts have been made to show a direct relationship between increases in synthesis of RNA resulting from administration of glucocorticoids and the induction of specific enzymes (for review see Tata, 1967a). In general these have relied on the effects of inhibitors, such as puromycin and actinomycin D, on the activities of the enzymes supposedly induced by the hormones. The inhibitors have widespread toxic effects in intact animals (Soeiro & Amos, 1966; Hofert & Boutwell, 1963). The pitfalls attendant on their use are demonstrated by the finding that in hepatoma tissue-culture cells actinomycin D exerts a profound influence on the rate of degradation of tyrosine transaminase (Reel & Kenney, 1968) and slows down the breakdown of microsomal RNA (Ottolenghi, Romano & Barnabei, 1966). The administration of cortisol seems to cause no change in the species of RNA synthesized in rat liver nor in their relative proportions. It may, however, accelerate the rate of transfer of nascent RNA from nucleus to cytoplasm (Finkel, Henshaw & Hiatt, 1966; Venkov, Angelov, Valeva & Hadjiolov, 1967). Among the cytoplasmic sites suggested for the action of cortisol are: the combination of mRNA with ribosomes (Gabourel & Comstock, 1964; Gould & Manner, 1967); the attachment of ribosomes to the endoplasmic reticulum (Pitot, Cho & Lamar, 1965); the rate of translation (Korner, 1960; Garren, Richardson & Crocco, 1967); and the folding of the nascent polypeptide chain (Griffin & Cox, 1966).

We have endeavoured to define the influence of cortisol on the structure and activity of the principal unit of the machinery for protein synthesis, the polyribosome, in the liver of rodents. For much of this work we have used mice because their smaller size results in higher specific radioactivities for a given dose in radioactivity experiments and individual variations can be limited by pooling livers of several animals.

MATERIALS AND METHODS

Animals. Mice were young adult (aged 6–10 weeks) male albinos of N.I.H. strain, weighing about 25g. Rats were albino Wistars, weighing 150–200g. They were housed in rooms with controlled lighting and were maintained on Rank's diet 41B and water *ad libitum*. When required, starvation was begun at 17.30hr. for a period of 16hr.

Radioactive materials. These were obtained from The Radiochemical Centre, Amersham, Bucks.

Assay for tryptophan pyrrolase. The procedure is based on that of Fiegelson & Greengard (1961). Pairs of animals were killed by cervical dislocation; the livers were removed, chopped up and homogenized in 7 vol. of ice-cold medium containing 0.14M-KCl, 2.5mM-NaOH and 1mM-L-tryptophan. Incubation was carried out at 37° in 250ml. conical flasks, which were shaken continuously in an atmosphere of air. The incubation mixture consisted of 0.1M-sodium phosphate buffer, pH 7.0 (30ml.), 0.04M-L-tryptophan (5ml.), 0.3M-ascorbic acid (freshly neutralized) (5ml.), water and homogenate (10ml.). Samples (4ml.) were removed at 15min. intervals over a period of 90min. and added to aq. 15% (w/v) metaphosphoric acid (2ml.). Then 3ml. of the filtered mixture was neutralized with 1.0M-NaOH and the E_{365} determined against a water blank. The activity was estimated from the slope of the plot of extinction against time by using the linear region between 30 and 90min. The activity is expressed in μ moles of kymurenine formed/hr./g. wet wt. of liver, by using a molar extinction coefficient of 4540. Addition of haematin, final concn. 3mM, slightly inhibited the reaction.

Kinetics of induction of tryptophan pyrrolase. Twelve mice were injected intraperitoneally with doses of hydrocortisone sodium succinate (Solu-Cortef; Upjohn Ltd., Crawley, Sussex) equivalent to 2.5mg. of cortisol/100g. body weight in 0.9% NaCl. At intervals of 2hr. pairs of animals were killed and the activity of tryptophan pyrrolase was determined in the pooled livers. Control animals were killed 4hr. after injection with 0.9% NaCl. The experiment was repeated twice.

Density-gradient centrifugation. Centrifugation was performed in 30ml. Spinco polyallomer tubes. Linear gradients were formed in a scaled-up version of the apparatus of Rosenbloom & Schumaker (1963) by using a pair of 20ml. glass-steel syringes (Summit, S. and R. J. Everett and Co. Ltd., Thornton Heath, Surrey) driven by a Palmer continuous-injector unit (Baird and Tatlock Ltd., Chadwell Heath, Essex). A glass rod was used to guide the gradient smoothly down the walls of the tube. Gradients were monitored as described by Edwards & Mathias (1963) with a quartz flow-cell (path length 2mm.) in a Cary model 14 recording spectrophotometer, at a constant flow rate of 1.5ml./min. Fractions were collected manually on a time basis.

Preparation of polyribosomes. Livers from starved animals were homogenized in 2 vol. of ice-cold 0.25M-sucrose in 50mM-tris-HCl (pH 7.6)–25mM-KCl–5mM-MgCl₂ (TKM) buffer in a Teflon-glass Potter-Elvehjem homogenizer with a clearance of 0.015in. by using eight strokes of the plunger. The homogenate was centrifuged at 16000g_{av.} for 10min. The supernatant was made 1.25% with respect to sodium deoxycholate. Samples (4ml.) were layered over 3ml. of 0.5M-sucrose in TKM buffer above 3.5ml. of 2.0M-sucrose in TKM buffer. After centrifugation at 105000g_{av.} for 4hr. at 0° the sucrose was drained off and the walls of the tubes were wiped dry. The pellets of polyribosomes were dissolved in 2ml. of the original medium by gentle shaking at 4°. Aggregated material was removed by a short centrifugation. The yields of polyribosomes were 2.2mg./g. wet wt. for rat liver and 1.95mg./g. wet wt. for mouse liver, assuming that a 1mg./ml. solution had E_{260} 11.2.

Preparation of total ribosomes. The preparation was

EXPLANATION OF PLATES 1 AND 2

All plates refer to mouse liver. The bar on each plate represents $1\mu\text{m}$.

1(a). Microsome fraction recovered from the interface between 0.5M- and 2.0M-sucrose. Both rough (R) and smooth (S) vesicles are shown. (b). Smooth-microsome fraction obtained from the interface between 0.25M- and 1.3M-sucrose. Some membranes bear ribosomes. However, the density of rough membranes is small compared with those shown in Plate 2(a).

2(a). Rough microsome fraction obtained from the interface between 1.3M- and 2.0M-sucrose. (b). Rough microsomes prepared from adrenalectomized animals. (c). Rough microsomes prepared from adrenalectomized animals that had received cortisol 2hr. before death.

identical with that of polyribosomes except the post-mitochondrial supernatant treated with deoxycholate was layered over 7 ml. of 1M-sucrose in TKM buffer. These preparations contain some ferritin (Wilson & Hoagland, 1965).

Separation of free and bound ribosomes. The procedure is based on that of Bloemendal, Bont & Benedetti (1964). The mouse liver postmitochondrial supernatant was isolated as in the experiments for the preparation of polyribosomes. Then 4 ml. of postmitochondrial supernatant was layered over 3.5 ml. of 0.5M-sucrose above 3.5 ml. of 2.0M-sucrose, both in TKM buffer. After centrifugation at 75000g_{av}. for 8 hr. at 1°, a pellet was visible and an orange layer was found at the interface of the 0.5M- and 2.0M-sucrose. This layer was recovered by aspiration in a syringe, made 1% with respect to sodium deoxycholate and diluted with an equal volume of 0.25M-sucrose in TKM buffer. A 7 ml. portion of this solution was layered over 3.5 ml. of 2.0M-sucrose in TKM buffer and centrifuged at 75000g_{av}. for 8 hr. at 1°. The pellet is designated 'bound ribosomes'. The pellet formed in the previous centrifugation is referred to as 'free ribosomes'. In normal mouse liver about two-thirds of the ribosomes are recovered as bound ribosomes. When the material in the orange layer was examined in gradients of the type devised by Tata (1967b) to identify microsomal components of tadpole liver, most of the material sedimented to the interface between the 30 and 60% (w/v) sucrose, where membranous fragments collect. Pretreatment with sodium deoxycholate eliminated this zone and gave rise to slower-sedimenting material with the characteristics of ribosomes and polyribosomes. Electron microscopy revealed that the orange layer contained both rough and smooth vesicles (Plate 1a).

Separation of rough from smooth microsomes. The procedure is based on those of Dallner (1963), Bloemendal, Bont, de Vries & Benedetti (1967) and Tata (1967b). The post-mitochondrial supernatant from a 1:2 (w/v) homogenate of mouse liver, prepared by centrifugation at 9500g_{av}. for 12 min., was diluted with 3 vol. of 0.25M-sucrose in TKM buffer, and 7 ml. was layered over 1.3M-sucrose in TKM buffer (7 ml.) above 2.0M-sucrose in TKM buffer (6 ml.). The tubes were centrifuged at 100000g_{av}. for 3 hr. at 2° in a 3 × 25 ml. swing-out rotor (Measuring and Scientific Equipment Ltd., London, S.W.1). The smooth microsomes concentrate at the interface between the 0.25M- and 1.3M-sucrose (Plate 1b) and the rough microsomes between the 1.3M- and 2.0M-sucrose (Plate 2a). The pellet contains polyribosomes and glycogen. Small amounts of the latter are found in the other fractions.

Kinetics of labelling of mouse liver polyribosomes. DL-[methylene-¹⁴C]Tryptophan (32.9 mc/m-mole) was diluted in 0.9% NaCl to give a concentration of 10 μC/ml. and injected intraperitoneally at a dose of 12 μC/100 g. body wt.

into mice weighing 25 g. that had been starved for 15 hr. Pairs of animals were killed at 2, 4, 6, 8 and 10 min. after injection, and polyribosomes were prepared and resuspended. A sample was removed for determination of the extinction at 280 nm. Radioactivity incorporated into protein was measured by the addition of 3 vol. of 10% (w/v) trichloroacetic acid. The precipitate was collected on Millipore filters (HAWP-025; Millipore Filter Corp., Bedford, Mass., U.S.A.) and washed with 5 vol. of trichloroacetic acid. The filters were dried, placed in vials containing 15 ml. of a solution of 0.4% (w/v) 2,5-bis-(5-tert.-butylbenzoxazol-2-yl)-thiophen (BBOT) [Ciba (A.R.L.) Ltd., Duxford, Cambs.] in toluene and counted in a Beckman liquid-scintillation counter for 100 min. or 10000 counts. Incubation of the polyribosome samples with 10% trichloroacetic acid at 90° for 30 min. caused no loss of radioactivity from the precipitate.

The distribution of radioactivity amongst polyribosomes was determined by layering a sample (1 ml.) obtained after 4 min. of labelling with L-[1-¹⁴C]leucine (8.3 μC/m-mole; 10 μC/100 g. body wt.) over a 10–40% (w/v) sucrose gradient in TKM buffer and centrifuging in the SW25.1 rotor at 63500g_{av}. for 2 hr. at 2°. The extinction profile at 260 nm. was monitored and the gradient collected in 22 equal fractions. To each fraction was added 1.0 ml. of a 0.05% solution of bovine serum albumin. (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) followed by 3 ml. of 10% trichloroacetic acid. After 30 min. at room temperature the precipitate was collected on Millipore filters and washed with 15 ml. of 10% trichloroacetic acid. The filters were dried and the radioactivity measured.

Effects of cortisol on the labelling of liver polyribosomes. Male adrenalectomized rats were starved for 16 hr. overnight. Two animals were injected with cortisol (2.5 mg./100 g. body wt.) 150 min. before being killed. A pair of control animals was injected with an equivalent volume of 0.9% NaCl. At 6 min. before death one animal from each pair received an injection of 40 μC of L-[1-¹⁴C]leucine (8.3 mc/m-mole) in a volume of 1 ml. and the other 400 μC of L-[³H]leucine (200 mc/m-mole). After the animals had been killed the livers were removed and divided into two equal portions. The halves of liver were combined according to the plan set out in Table 1. Polyribosomes were isolated and analysed by density-gradient centrifugation in the SW25.1 rotor by using a linear 10–40% (w/v) sucrose density gradient (63500g_{av}. for 2 hr. at 1°). The gradients were collected in fractions (1.3 ml.) and the radioactivity was estimated by the gel method described below.

Determination of radioactivity in samples containing ¹⁴C and ³H from sucrose-density gradients. Samples (up to 0.5 ml.) containing sucrose (maximum concentration 40%, w/v) were pipetted into glass vials. Then 1.5 ml. of hot (95°) solution (1.4%) of agarose [BDH (Chemicals) Ltd., Poole,

Table 1. Double-labelling experiment to assess the effects of cortisol on liver polyribosomes

Portions of liver designated with the same letter were mixed before preparation of polyribosomes.

Label used	¹⁴ C	+	+	+	+				
	³ H					+	+	+	+
Treatment	Cortisol	+	+			+	+		
	Saline			+	+			+	+
Designation		B	D	A	C	A	D	B	C

Dorset] was added with gentle shaking. The final concentration of sucrose in the gel must not exceed 10% (w/v), otherwise formation of sucrose crystals occurs. If this problem does not arise, the volume of the gel may with advantage be decreased below 2 ml. After gelation 15 ml. of scintillation fluid was added containing (per l.) 4 g. of BBOT, 80 g. of naphthalene, 400 ml. of 2-methoxyethanol and 600 ml. of toluene. Water was leached from the gel by rotating the vials on a slow-moving turntable for 2 days with two changes of scintillation fluid. After a final change of scintillation fluid the gels were completely translucent. The vials were counted in a Beckman scintillation counter. Trial experiments showed that there was a linear response to both ^{14}C and ^3H labels in polyribosomes or microsomes with a load up to at least the equivalent of 0.3 mg. of RNA. With purified radioactive polyribosome preparations, less than 2% of the radioactivity was lost during the leaching process. If the radioactivity of a postmitochondrial supernatant fraction prepared from the liver of animals that had received a 6 min. pulse of radioactive amino acids was measured in this way, $59.4 \pm 0.7\%$ of the ^{14}C counts and $58.4 \pm 0.3\%$ of the ^3H counts were lost during the leaching stage. Increasing the number of changes of scintillation fluid caused no additional fall in the radioactivity of the gels. In the Millipore technique only 27% of the ^{14}C or ^3H counts were retained from the same sample. The agarose gel appears to trap small peptides not precipitated by trichloroacetic acid and not retained by the filters. Quench, as determined by the ratio of counts in two external standard channels, was unaffected by sucrose concentration and varied only to a minor random degree along a gradient or between gradients. A programme written for an Olivetti Programma 101 computer was used to calculate ^3H and ^{14}C d.p.m. and their ratio for each sample. For comparison of radioactivities of samples in a gradient the ratio of individual fractions was normalized by dividing it by the ratio of total ^3H d.p.m. to total ^{14}C d.p.m. recovered, and is referred to as the excess ratio.

Double labelling and fractionation of rough microsomes. Six male mice were used 7 days after adrenalectomy. At 150 min. before death, one pair was injected with cortisol (2.5 mg./100 g. body wt.) and the remainder with an equivalent volume of 0.9% NaCl. At 6 min. before death three animals selected according to the plan in Table 2 were injected with $10\mu\text{C}$ of ^{14}C -labelled amino acids in 1 ml. of solution made up as follows: L-alanine, $2\mu\text{C}$, $1\mu\text{mole}$; L-aspartic acid, $2\mu\text{C}$, $1\mu\text{mole}$; glycine, $2\mu\text{C}$, $1\mu\text{mole}$; L-leucine, $2\mu\text{C}$, $1\mu\text{mole}$; L-3-phenylalanine, $2\mu\text{C}$, $1\mu\text{mole}$. The remaining animals received $92\mu\text{C}$ of ^3H -labelled amino acids, also in 1 ml.: L-alanine, $20\mu\text{C}$, $1\mu\text{mole}$; L-aspartic acid, $20\mu\text{C}$, $1\mu\text{mole}$; glycine, $20\mu\text{C}$, $1\mu\text{mole}$; L-leucine, $16\mu\text{C}$, $1\mu\text{mole}$; L-3-phenylalanine, $16\mu\text{C}$, $1\mu\text{mole}$. After the animals had been killed the livers were removed and each liver was adjusted to 1.4 g. They were combined according to the plan in Table 2. The rough microsomes

were prepared and collected in a volume of 2 ml. They were diluted to 6 ml. with 0.25 M-sucrose in TKM buffer. Samples (2 ml.) were layered over a discontinuous gradient consisting of 2.0 M-sucrose (4 ml.), 1.6 M-sucrose (13 ml.), 1.4 M-sucrose (5 ml.) and 1.3 M-sucrose (5 ml.), all in TKM buffer. After centrifugation in the SW25.1 rotor at $51000g_{\text{av}}$ for 16 hr. at 2° , the gradients were monitored and collected in 20 fractions of equal volume, which were counted by the agarose-gel technique.

Amino acid pools of mouse liver. Four adrenalectomized mice were divided into two pairs. One pair was injected with 0.2 ml. of a cortisol solution equivalent to 2.5 mg./100 g. body wt. A pair of unoperated animals injected with 0.9% NaCl served as a control. Then 5 hr. later each animal was given an injection (1 ml.) of a mixture of ^{14}C -labelled amino acids: L-alanine (75 mc/m-mole), $2\mu\text{C}$; L-aspartic acid (100 mc/m-mole), $2\mu\text{C}$; L-leucine (150 mc/m-mole), $2\mu\text{C}$; L-lysine (150 mc/m-mole), $2\mu\text{C}$; L-3-phenylalanine (225 mc/m-mole), $20\mu\text{C}$. Then 2 min. afterwards the animals were killed and the livers removed with tongs previously frozen in liquid N_2 . The amino acids were isolated by the method of Wollenberger, Ristau & Schoffa (1960) and samples analysed on a Technicon amino acid analyser. The effluent from the ion-exchange column was divided into two equal streams. One was treated with ninhydrin and the other collected in a fraction cutter. About 100 fractions were collected for each analysis. Samples (0.5 ml.) of each fraction were mixed with 15 ml. of the scintillation fluid used for the gel experiments and counted to 10000 counts.

RNA. This was determined by the modification of the Schmidt-Thannhauser method devised by Fleck & Munro (1966) by assuming that E_{260} 1.00 is equivalent to $32\mu\text{g}$. of RNA/ml.

Electron microscopy. The method was based on that used by Dallner (1963). Microsomal fractions were collected as pellets by centrifugation at $100000g$ for 1 hr. at 2° . The pellets were fixed for 15 hr. at 4° in 1% osmium tetroxide adjusted to pH 7.4 with sodium phosphate buffer. The pellets were cut into small pieces, dehydrated by passage through graded ethanol solutions and embedded in Araldite. Sections were cut at a thickness of approx. $600\text{\AA}$ by using a Porter-Blum MT-2 ultramicrotome and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Our first task was to investigate the time-course of the induction by cortisol of tryptophan pyrrolase, which, although firmly established in rat liver (Mishkin & Shore, 1967), was undefined in mice. Fig. 1 shows that the activity begins to increase 2 hr. after the injection of cortisol. It reaches a maximum value, approx. 8 hr. after the administration of the hormone, of three times that of control

Table 2. *Double-labelling experiment to assess the effects of cortisol on rough microsomes*

Label	^{14}C	+	+	+	+	+	+
	^3H						
Treatment	Cortisol			+			+
	Saline	+	+		+	+	
Designation		A	C	B	B	C	A

Fig. 1. Kinetics of induction of tryptophan pyrrolase in mouse liver. Twelve mice were injected intraperitoneally with doses of hydrocortisone sodium succinate (Solu-Cortef) equivalent to 2.5 mg. of cortisol/100 g. body weight in 0.9% NaCl. At 2 hr. intervals pairs of animals were killed and the activity of tryptophan pyrrolase was determined in the pooled livers. Control animals were killed 4 hr. after injection with 0.9% NaCl. The experiment was repeated twice. Vertical bars represent standard deviation of the mean.

animals. Within a further 4 hr. it returned nearly to normal. Apart from the fact that the peak value is reached 1–2 hr. later in mice than in rats, the induction process is similar in both species. Starvation for 16 hr. had no effect on the activity of tryptophan pyrrolase, which, however, was decreased to one-third of the normal value in animals that had been adrenalectomized 6 days before removal of the liver.

A second preliminary investigation concerned the kinetics of labelling of nascent protein in polyribosomes. A typical experiment is shown in Fig. 2. Maximum specific radioactivity is attained within 6–8 min. Experiments with rats gave similar results. The pattern of incorporation of amino acid into various sizes of polyribosomes is shown in Fig. 3. A remarkable feature of this profile is the significant increase of specific radioactivity as the number of ribosomes in the polyribosome increases. This indicates that the average length of the nascent polypeptide chain is dependent on the number of ribosomes in the polyribosome. Kuff &

Fig. 2. Kinetics of labelling of nascent protein in polyribosomes from mouse liver. Mice weighing 25 g. each received 3 μC of DL-[methylene-¹⁴C]tryptophan intraperitoneally at appropriate times before death. Animals were killed in pairs and polyribosomes prepared from the livers.

Roberts (1967) have demonstrated in mouse tumours a linear relationship between specific radioactivity and size of polyribosome. They conclude that in these cells there can be no internal initiation of protein synthesis. This implies that the mRNA is monocistronic (Jacobson & Baltimore, 1968). The general character of this observation, now extended to rodent liver tissue, suggests that most if not all mammalian mRNA species are monocistronic.

Effect of cortisol on the incorporation of amino acid by subcellular fractions. Fig. 4 shows the effects of cortisol on the incorporation of amino acids into protein in various fractions derived from the livers of mice that had been adrenalectomized 6 days before the experiment. Three pairs of male adrenalectomized animals were injected with cortisol (2.5 mg./100 g. body wt.) at 2½, 5 and 7½ hr. before death. At 2 min. before death each animal received 9 μC of ¹⁴C-labelled protein hydrolysate. The subcellular fractions were isolated by the methods described in the Materials and Methods section. In a parallel experiment the effects of cortisol on the activity of tryptophan pyrrolase were determined. Adrenalectomy impairs to a slight extent the efficiency of incorporation in a 2 min. pulse by the subcellular fractions engaged in protein synthesis. Administration of cortisol increases their activity to a value at least six times that obtained in control animals. Concomitant with the enhanced incorporation, the hormone elevates the activity of tryptophan pyrrolase in adrenalectomized mice,

Fig. 3. Profile of incorporation of [^{14}C]leucine into nascent protein of liver polyribosomes. Mouse liver polyribosomes were labelled for 4 min. with L-[^{14}C]leucine and centrifuged in a gradient of 10–40% (w/v) sucrose in TKM buffer at 63500g for 2 hr. at 2°. Fractions (1.3 ml.) were collected. —, E_{280} ; ○, ^{14}C radioactivity; △, specific radioactivity.

restoring it within 6 hr. to that of control animals. Essentially similar results were obtained if the pulse time was extended to between 4 and 10 min.

Specificity of the stimulation of incorporation by cortisol. The increases in the rate of incorporation of amino acids into several subcellular fractions could arise either from a general increase of protein synthesis in the liver, or because of specific selective increases in the rate of synthesis of a small number of proteins. The second alternative could be achieved either by an enhanced production of the mRNA species corresponding to these proteins or by a discrimination by the machinery of protein synthesis for preformed mRNA species corresponding to these proteins. Either circumstance would be revealed by an increased incorporation of amino acids into certain regions of the profile of polyribosomes. We have therefore compared the nascent proteins attached to polyribosomes from the livers of adrenalectomized animals treated with cortisol with the pattern from untreated animals. To eliminate minor incidental variations between individual polyribosome preparations and their analyses by sucrose-density-gradient centrifugation, we have employed a double-labelling technique in which livers from animals receiving different

Fig. 4. Increases (a) in the activity of tryptophan pyrrolase and (b) in the efficiency of various subcellular fractions in the incorporation of radioactive amino acids into protein in livers of adrenalectomized mice treated with cortisol. At 2 min. before death each animal received $9\mu\text{C}$ of ^{14}C -labelled protein hydrolysate (54 mc/mg. atom of C). The post-mitochondrial supernatant from the pooled livers of each set was diluted to 8 ml. with 0.25M-sucrose in TKM buffer. Half was used for the preparation of free ribosomes (□) and bound ribosomes (○). The other half was used for the preparation of total ribosomes (△) and total polyribosomes (▲). Each fraction was analysed for RNA and radioactivity insoluble in trichloroacetic acid. The values for the livers of a pair of normal animals were: total polyribosomes 1515, total ribosomes 2010, bound ribosomes 2120 and free ribosomes 895 c.p.m./mg. of RNA.

treatments were mixed and in all subsequent stages subjected to exactly the same conditions. The design of these experiments is set out in the Materials and Methods section, under 'Effect of cortisol on labelling of liver polyribosomes'. An examination of the profiles of Expt. A and its reverse Expt. B (see Table 1), and their comparison with the control Expts. C and D, failed to reveal any deviation in Expt. A that was reversed in Expt. B and significantly different from the variation of the excess ratio in Expts. C and D. However, there is a trend of the excess-ratio value in Expt. A from a value greater than 1 to a value less than 1 in going from the top to the bottom of the gradient, and this is reversed in Expt. B. This suggests that in animals treated with cortisol the smaller polyribosomes are more active than those in the corresponding region from control animals. The effect is a small one and

Fig. 5. (a) Double-labelling experiment showing the effects of cortisol on the incorporation of amino acids in rough microsomes. In this experiment the animals treated with cortisol received the ${}^{14}\text{C}$ -labelled amino acids ($\circ$) and the control animals ${}^3\text{H}$ -labelled amino acids ($\square$). This corresponds to set B in Table 2. The full line is the profile of light-scattering at 260 nm. The lower diagram shows the excess-ratio value, i.e. $({}^3\text{H}/{}^{14}\text{C})/(\Sigma {}^3\text{H}/\Sigma {}^{14}\text{C})$. Δ , Results from set B; $\blacktriangle$, results from set C, in which both animals received an injection of 0.9% NaCl. (b) Reversal of labels with ${}^{14}\text{C}$ in control and ${}^3\text{H}$ in the cortisol-treated animals (set A).

may lie within experimental error. Certainly cortisol has no clear-cut effect on an individual class of polyribosomes.

Role of the endoplasmic reticulum in mediating the

effects of cortisol. The demonstration that there was no convincing effect of cortisol at the level of the polyribosome led us to investigate the possibility that the hormone influenced the binding of ribosomes to the membrane of the endoplasmic reticulum. The role of the endoplasmic reticulum in regulating the rate of protein synthesis has been discussed by Campbell (1964). The stage of development has a profound influence on these structures (Dallner, Siekevitz & Palade, 1965), which are also affected by the diet and the administration of drugs (Kato, Loeb & Gelboin, 1965; Orrenius, Ericsson & Ernster, 1965). We endeavoured to display in a gradient the range of vesicles in the fraction of the liver homogenate designated as the rough endoplasmic reticulum. These vesicles are characterized by a broad range of sedimentation coefficients and densities (Rothschild, 1963; Anderson, 1966). They may be separated isopycnicly into two broad peaks. By using a mixture of amino acids as a tracer, a double-labelling experiment was designed to investigate the possibility that cortisol modified the activity in protein synthesis of any part of the spectrum of rough vesicles. The experimental design is set out in Table 2 and the results are shown in Fig. 5. The excess ratio in the control experiment is close to unity throughout the gradient. This establishes the absence of counting artifacts and gives a measure of the variability to be expected between individual animals. The trend in ratio value from a region of ${}^{14}\text{C}$ excess at the bottom of the gradient to ${}^3\text{H}$ excess at the top of the gradient when animals that had received the hormone were given the ${}^{14}\text{C}$ -labelled amino acids (Fig. 5a) is clearly reversed when the labels are exchanged (Fig. 5b). The ratio values are considerably greater than the limits of experimental error found in the control gradient. This experiment indicates that as a result of the administration of cortisol the denser vesicles have a greater ability to incorporate amino acids relative to the light vesicles. This effect could arise from several causes. Either the hormone enhances the rate of protein synthesis in those regions of the endoplasmic reticulum that give rise to vesicles of high density during homogenization, or the hormone causes an increase in the formation of membranes densely packed with ribosomes with the result that homogenization produces a larger proportion of dense vesicles. The former possibility appears to be the less likely one in view of the evidence from the previous experiment suggesting that cortisol does not have a selective effect on the transcription or translation of mRNA.

Effects of cortisol on the amino acid pools in liver.

Chambers, Georg & Bass (1965) demonstrated that cortisol increases the uptake of α -aminoisobutyric acid by rat liver. Schimke *et al.* (1965) showed that the time-course of disappearance of radioactive

Table 3. *Effects of adrenalectomy and cortisol on the amino acid pools of mouse liver*

Average values from two experiments are given. Abbreviations: Ax, adrenalectomized animals; Ax+C, adrenalectomized animals treated with cortisol.

	Concn. of amino acid (μ moles/g. of liver)			Radioactivity in peak (c.p.m.)			$10^{-3} \times$ Sp. radioactivity (c.p.m./ μ mole/g. of liver)		
	Normal	Ax	Ax+C	Normal	Ax	Ax+C	Normal	Ax	Ax+C
Ala	2.14	1.24	2.25	9090	7329	10055	4.25	5.91	4.51
Asp	0.65	0.51	0.66	5243	3725	4682	8.06	7.3	7.09
Gly	2.14	1.61	2.01						
Glu	2.10	2.04	2.70	554	508	933			
Glu	2.01	2.04	2.70						
Ile	0.08	0.15	0.07						
Leu	0.23	0.23	0.21	13261	10805	12522	5.66	4.69	5.96
Lys	0.50	0.70	0.74	15655	12515	20925	31.3	17.9	28.0
Met	0.08	0.05	0.06						
Phe	0.12	0.09	0.13	14330	11155	13881	119	124	106
Tyr	0.10	0.13	0.07						
Val	0.29	0.37	0.29						

leucine from the liver pool was not altered by cortisol. However, it has been reported that high doses of triamcinolone and cortisone cause a rapid increase in the amino acid nitrogen in rat liver (Weber, Srivastava & Singhal, 1965). Radioactive amino acids have been used as tracers in many of the experiments reported in the present paper. We have therefore measured the effects of adrenalectomy and cortisol both on the amino acid pools of the liver and on the specific radioactivity of labelled amino acids injected intraperitoneally. These experiments afford a measure of the extent to which cortisol aids the passage of amino acids from the peritoneal cavity to the liver, minimizes their dispersal to other tissues and affects their transport across the membrane of the liver cell. The results are shown in Table 3.

Adrenalectomy causes a sharp fall in the concentration of alanine, but much less marked changes in other amino acids, some of which (e.g. lysine, valine) are actually increased. In general, the effect of the hormone is to restore the amino acid pools of adrenalectomized animals to values similar to those of normal animals.

Adrenalectomy causes a decline in the amounts transported, from the peritoneal cavity to the liver during the 2 min. after injection, of all the radioactive amino acids that we have examined. Administration of cortisol boosts this to values comparable with or exceeding those found in normal animals. An interesting observation is that for all categories of animals a small amount of radioactivity is found in association with the glutamic acid peak, even though this amino acid was not included in the labelled series used for injection.

In considering the incorporation of labelled amino acids it is the specific radioactivities of the liver pools

that is of primary importance. It is clear from Table 3 that, although the hormone does cause changes in this parameter, these are small and occur in opposite senses. Some, e.g. leucine and lysine, increase; others (alanine and phenylalanine) decrease. It is very doubtful if they are sufficient to account for changes we have observed in the labelling patterns of the subcellular fractions.

Effect of cortisol on the distribution of ribosomes in the cytoplasm. The tentative conclusion from the experiments on the double-labelling of the rough microsomes that cortisol influenced the binding of polyribosomes to membranes is in agreement with the suggestion of Peraino, Lamar & Pitot (1966). We have measured the percentage of ribosomes present as polyribosomes and the distribution of the particles between the bound and the free form in normal mouse liver. The results have been compared with those obtained from adrenalectomized animals before and after injection of cortisol. Two experiments were performed, each involving 20 mice. Four unoperated animals formed the control set. Sixteen adrenalectomized male mice were arranged in four sets of four. The animals in three of these sets received cortisol (2.5 mg./100 g. body wt.) 2½, 5 and 7 hr. before death. In one experiment the polyribosomes and total ribosomes were measured and the percentage of ribosomes as polyribosomes was calculated. In the other the percentage of free and bound ribosomes was determined. The results are shown in Table 4.

At 6 days after adrenalectomy there is a significant increase in the percentage of ribosomes in the form of polyribosomes, but the percentage of ribosomes bound to the endoplasmic reticulum has fallen from approx. 70% to 60%. Injection of cortisol has complex effects. Initially it increases

Table 4. *Effects of adrenalectomy and treatment with cortisol on the distribution of ribosomes in mouse liver*

The results are given as means $\pm$ S.E.M. The values in adrenalectomized animals are significantly different from normal animals at $P < 0.05$ (Student's *t* test). The values in adrenalectomized animals treated with cortisol that differ significantly ($P < 0.05$) from those in adrenalectomized animals are marked with an asterisk.

Treatment	% of ribosomes as poly-ribosomes	% of ribosomes bound to endoplasmic reticulum
Normal	59 $\pm$ 3.2	69 $\pm$ 2.7
Adrenalectomized	78 $\pm$ 1.3	60 $\pm$ 1.2
Adrenalectomized + cortisol (2.5 hr.)	81 $\pm$ 4.7	82 $\pm$ 3.8*
Adrenalectomized + cortisol (5 hr.)	68 $\pm$ 1.5*	73 $\pm$ 2.0*
Adrenalectomized + cortisol (7 hr.)	72 $\pm$ 3.5	78 $\pm$ 4.4

the percentage of polyribosomes. Subsequently the proportion of single ribosomes increases and the percentage of the ribosomes found as polyribosomes shows a tendency to shift nearer to that of normal animals. Perhaps the most marked change wrought by treating adrenalectomized animals with cortisol is the increase in the percentage of the ribosomes bound to the endoplasmic reticulum. This changes from 60 to 82% within 2½ hr. after administration of the hormone. Even 7 hr. afterwards the value is above that of normal animals. The results clearly indicate that removal of the adrenals causes a decrease in percentage of bound ribosomes, which is reversed by injection of cortisol.

Direct confirmation of this phenomenon was obtained by electron microscopy. Examination of the rough microsomes from normal animals (Plate 2a) reveals that the ribosomes are tightly bound to the periphery of most of the vesicles and evenly distributed over the surface. In contrast the ribosomes in the rough-microsome fraction from adrenalectomized mice (Plate 2b) appear to be much more loosely attached to the membrane. The density of packing is less regular. At 2 hr. after treatment with cortisol (Plate 2c) there is an obvious increase in the closeness of attachment of the ribosomes to the vesicles and in the density and evenness of their packing.

DISCUSSION

The strong induction of tryptophan pyrrolase by cortisol in rodent liver has been observed for one other enzyme, tyrosine transaminase (Holten & Kenney, 1967), which is also characterized by a

short half-life (Kenney, 1967). The fact that the increase in enzymic activity is preceded by an elevation of RNA synthesis (Kenney & Kull, 1963; Drews & Brawerman, 1967) has led to speculation that the two processes are related, the burst of enzyme synthesis following an increased production of specific mRNA molecules promoted by the hormone.

Our experiments cannot rule out this possibility. The failure to detect enhanced activity of a specific class of polyribosomes may arise from the lack of sufficient sensitivity in the method. However, two effects of cortisol on the complex systems for protein synthesis have been established, a decline in the percentage of ribosomes in the form of polyribosomes and an increase in the percentage of total ribosomes bound to the endoplasmic reticulum. Franklin & Godfrey (1966) have also found that cortisone caused an accumulation of monoribosomes in rat liver. Our estimate for the percentage of ribosomes that are bound to membranes in normal animals agrees with that of Webb, Blobel, Potter & Morris (1965). The enhancement of binding brought about by cortisol is reminiscent of the effect of administering thyroid hormone to thyroidectomized rats, although this is accompanied by an increase in the average size and amount of polyribosomes (Tata & Widnell, 1966).

The nature of the attachment of the ribosome to the endoplasmic reticulum is unknown. It appears to involve the larger subunit (Sabatini, Tashiro & Palade, 1966; Shelton & Kuff, 1966). The free and bound polyribosomes are not significantly different, but the membrane of the rough endoplasmic reticulum appears to have a greater capacity to bind polyribosomes compared with the smooth (Suss, Blobel & Pitot, 1966). Cortisol does not exert a selective effect on the rate of amino acid incorporation in bound ribosomes (Fig. 4) in the sense that both bound and free ribosomes are stimulated to approximately the same extent. This indicates that the effect of cortisol is on the membrane rather than the ribosomes. Possibly the hormone or a metabolite brings about the formation of specific binding sites on the membrane. Alternatively it could assist the removal of blocking agents that prevent the attachment of ribosomes to membranes.

The influence of cortisol on RNA synthesis may be linked with its tendency to bind to histones both *in vivo* and *in vitro* (Salganik, Morozova & Zakharov, 1968; Sluyser, 1966). In principle this could affect both the rate and type of RNA synthesis. If in addition the hormone promoted a more rapid transfer of newly synthesized RNA from the nucleus to the cytoplasm (Finkel *et al.* 1966; Venkov *et al.* 1967), in which there was an element of discrimination accomplished by selective binding to membranes (Tata, 1968), apparent induction of specific

enzymes would ensue. The binding of the poly-ribosomes to membranes might also result in a longer half-life for the mRNA, which in itself would result in an alteration of the balance of enzymes.

The possibility exists that the mRNA-ribosome complex is attached to newly synthesized membrane as it emerges from the nucleus. There is RNA ranging in size from 6s to 20s associated with both rough and smooth membranes (Tata, 1967c) that is quite distinct from the 7s RNA of the larger ribosomal subunit (Pene, Knight & Darnell, 1968). The rate of labelling of the membrane-associated RNA is enhanced by hormonal treatment. The relation of the elevated binding of ribosomes to membrane in the presence of cortisol and this novel form of membrane RNA is unknown. Our experiments clearly indicate that cortisol or a metabolite interacts with the membrane to generate an increased number of ribosome-binding sites. Studies of systems *in vitro* lend support to the postulate that steroids are involved in the attachment of ribosomes to the membrane of the endoplasmic reticulum (Williams & Rabin, 1969).

Note added in proof. Mills & Toppes (1969) have shown that cortisol is necessary for the formation of rough endoplasmic reticulum in mouse mammary alveolar epithelial cells.

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