Protein Synthesis by Brain-Cortex Mitochondria

CHARACTERIZATION OF A 55S MITOCHONDRIAL RIBOSOME AS THE FUNCTIONAL UNIT IN PROTEIN SYNTHESIS BY CORTEX MITOCHONDRIA AND ITS DISTINCTION FROM A CONTAMINANT CYTOPLASMIC PROTEIN-SYNTHESIZING SYSTEM

BY A. HERNANDEZ, I. BURDETT AND T. S. WORK

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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Homogenates of rat brain cortex were fractionated by conventional methods of velocity sedimentation and separated into a microsomal and a washed mitochondrial fraction. By electron microscopy the mitochondrial fraction was shown to be rich in synaptosomes. The mitochondria-synaptosome fraction synthesized protein *in vitro* by a route that was partially inhibited by cycloheximide and partly by chloramphenicol. The relative effectiveness of the two inhibitors varied greatly with the medium used. In the mitochondria-synaptosome fraction active 80S cytoplasmic ribosomes and active 55S mitochondrial ribosomes were detected; these were also seen in the electron microscope. Mild osmotic shock of the mitochondria-synaptosome fraction followed by velocity sedimentation in sucrose-EDTA allowed isolation of a mitochondrial fraction free of synaptosomes. Protein synthesis in this fraction was entirely inhibited by chloramphenicol, but was completely resistant to cycloheximide both in a medium promoting oxidative phosphorylation and in ATP-generating medium. Ouabain had no inhibitory effect on protein synthesis in a purified mitochondrial preparation. It is concluded that brain-cortex mitochondria synthesize protein entirely on 55S mitochondrial ribosomes.

The highly specialized neuronal cells of the brain communicate through synaptic processes. The synaptic process at its peripheral end terminates in a synaptic knob, which is filled with synaptic vesicles and mitochondria (Gray & Guillery, 1966). This region of the nerve cell requires considerable quantities of protein in order to carry out its specialized functions, but it has not been shown to contain ribosomes. When cerebral cortex is homogenized in a Potter homogenizer the synaptic knobs are torn from their parent cells and round up into osmotically fragile organelle-like bodies known as synaptosomes (Whittaker, 1965). Over the past seven years protein synthesis by synaptosomal fractions has been studied in many laboratories but the results have been confusing and at times contradictory. There is general agreement that these synaptosomal preparations are capable of extensive protein synthesis (Morgan & Austin, 1968; Autilio, Appel, Pettis & Gambetti, 1968) but in the absence of recognizable ribosomes within the synaptic particles there has been disagreement as to how this synthesis is mediated. It has been proposed either that synaptosomes contain a specialized

membrane-RNA capable of protein synthesis (Morgan & Austin, 1968) or that synaptosomal mitochondria are specialized so as to permit them to perform a range of protein synthesis that does not occur in mitochondria elsewhere in the body (Gordon & Deanin, 1968; Cunningham & Bridgers, 1970).

The hypothesis that neuronal cells contain specialized mitochondria was strongly supported by the observation of Gordon & Deanin (1968) that washed mitochondria from brain-cortex synthesized protein by a route that was more sensitive to cycloheximide than to chloramphenicol. Mitochondria from other tissues synthesize protein by a mechanism that is readily inhibited by chloramphenicol and completely resistant to cycloheximide (see review by Ashwell & Work, 1970b) whereas cycloheximide inhibition is normally regarded as diagnostic for synthesis on 80S ribosomes. The hypothesis that there is a specialized cortex mitochondrion has been questioned by several laboratories (Campbell, Mahler, Moore & Tewari, 1966; Morgan, 1970; Haldar, 1970; Bosmann & Hemsworth, 1970) but it has not hitherto been possible to

prepare brain-cortex mitochondria that synthesize protein by a route entirely resistant to cycloheximide.

In the present investigation we have prepared from brain-cortex homogenates a fraction consisting mainly of mitochondria and synaptosomes. This preparation synthesized protein by a route that was strongly inhibited by cycloheximide, partially inhibited by chloramphenicol and contained 80S ribosomes and polyribosomes. In purified mitochondrial preparations we detected a 55S mitochondrial ribosome active in protein synthesis, sensitive to chloramphenicol and resistant to cycloheximide.

In our opinion previous confusion in this field has been due to the difficulty of obtaining mitochondria free of contamination by 80S ribosomes.

MATERIALS AND METHODS

Reagents. Radioactive L-leucine (344 mCi/mmol), L-isoleucine (312 mCi/mmol) and L-valine (260 mCi/mmol), uniformly labelled with ^{14}C , were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Strophanthin G (ouabain) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The ADP, ATP, pyruvate kinase, phosphoenolpyruvate and ribonuclease were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The bovine plasma albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Chloramphenicol was from Parke Davis and Co. Ltd., Hounslow, Middx., U.K., and cycloheximide and sodium deoxycholate were purchased from Koch-Light. Brij-58 was from Honeywell-Atlas Ltd., Carshalton, Surrey, U.K. All other reagents were of A.R. quality.

Tissue homogenates. Male Sprague-Dawley rats were killed by a sharp blow on the neck. The brain was excised within 1 min and chilled in saline (0.121 M-NaCl-0.024 M-KCl). The cerebral cortex was separated from the white matter and forced through a stainless-steel tissue mincer of the type described by Porterfield (1960). The mince (1 vol.) was mixed with 4 vol. of either ice-cold 0.3 M-sucrose-10 mM-tris-HCl, pH 7.4, or 0.3 M-sucrose-0.5 mM-EDTA, adjusted with KOH to pH 7.4. When microsomal fractions were to be prepared, the cortex was processed as described above, but in ice-cold 0.25 M-sucrose-25 mM-KCl-4 mM-MgCl₂-10 mM-tris-HCl, pH 7.4. Homogenization was carried out in a Potter-Elvehjem-type homogenizer at 0°C with a 3.2 cm-diam. polytetrafluoroethylene (Teflon) pestle (clearance 0.48–0.72 mm as measured on the diameter) with five to eight slow strokes, the pestle rotating at 1500–2000 rev./min, and the final homogenate was made 10% (w/v) with the appropriate isolation medium.

Mitochondria-synaptosome mixture. The sucrose homogenate in either 0.3 M-sucrose-10 mM-tris-HCl, pH 7.4, or 0.3 M-sucrose-0.5 mM-EDTA, pH 7.4, was centrifuged at 700g for 15 min and the supernatant carefully removed. The pellet was resuspended in one-third the original volume of the appropriate sucrose mixture and re-centrifuged at 700g. The combined supernatants were centrifuged at 12000g for 15 min at 0–4°C in the 69181

angle rotor of the MSE 18 centrifuge. All subsequent centrifugations were made at 0–4°C in the same centrifuge, except when otherwise stated. The pellet was resuspended in one-half the original volume of isolation medium and centrifuged at 7000g for 15 min. The pellet was again resuspended in one-third the volume of isolation medium and centrifuged at 9000g for 15 min and the 'fluff' material above the firm pellet was discarded. To remove residual nuclei the pellet was resuspended in the appropriate isolation medium (one-third of original volume) and centrifuged again at 700g for 10 min. The supernatant was carefully aspirated off so as to avoid disturbance of any residual nuclei or whole cells; the supernatant was centrifuged again at 9000g for 15 min. The pellet obtained in this step was used either to prepare the purified mitochondrial fraction as described later or washed at 9000g once more. This material, which consists mainly of synaptosomes and mitochondria, will be referred to in this paper as the mitochondria-synaptosome mixture obtained in 0.3 M-sucrose-10 mM-tris-HCl, pH 7.4, or in 0.3 M-sucrose-0.5 mM-EDTA, pH 7.4.

Purified mitochondria. The pellet obtained by use of 0.3 M-sucrose-0.5 mM-EDTA, pH 7.4, as described above, was mixed gently (by Pasteur pipette) with ice-cold water (1.25 ml/g of original cortex) and after 5 min of gentle agitation, the osmotic pressure of the medium was raised by adding bovine plasma albumin (10 mg/ml) followed by 0.3 M-sucrose-0.5 mM-EDTA.

For each 1.0 ml of water used, we added 0.2 ml of albumin and 0.8 ml of sucrose-EDTA. After gentle mixing, the suspension was spun at 9000g for 10 min and the 'fluffy' layer above the firm brown pellet was discarded. The pellet was resuspended in the appropriate incubation medium and used immediately. The yield of this fraction was 1.0–1.5 mg of protein/g of fresh cortex.

Microsomal fraction. Cell debris, nuclei, synaptosomes and mitochondria were sedimented by centrifuging a cortex homogenate (0.25 M-sucrose-25 mM-KCl-4 mM-magnesium acetate-10 mM-tris-HCl, pH 7.6) at 12000g for 20 min at 4°C. The supernatant fluid was removed and centrifuged at 105000g for 2 h at 2°C in the Spinco model L preparative ultracentrifuge to sediment the total microsomal fraction. The microsomal fraction obtained was rinsed twice with the isolation medium and drained by inverting the centrifuge tube. This fraction when not used immediately was stored at –20°C overnight. The yield of this fraction was 3 mg of protein/g of fresh cortex.

pH 5 enzyme. This fraction was obtained from the post-microsomal supernatant (Keller & Zamecnik, 1956) by adjusting the pH to 5 with 1 M-acetic acid and centrifuging the cloudy solution at 17000g for 20 min at 0–4°C. The pellet was resuspended in the microsomal incubation medium and used within 24 h.

Incubation media. Three incubation media were used.

Medium A had the following composition (final concentrations): 0.3 M-mannitol, 10 mM-K₂HPO₄, 10 mM-tris-HCl, pH 7.6, 10 mM-magnesium acetate, 5 mM- α -oxoglutaric acid, 0.1 mM-EDTA, 1 mM-ADP, 0.05 μM of each of the 20 amino acids except leucine as recommended by Roodyn, Reis & Work (1961). To each 1 ml of this medium was added 1 mg of bovine plasma albumin and 0.2 mg of cytochrome c. In some experiments α -oxoglutaric acid and ADP were not added and the energy was supplied by an ATP-generating system containing

2 mM-ATP, 5 mM-phosphoenolpyruvate and 10 μ g of pyruvate kinase/ml. This last medium is referred to below as the ATP-generating system.

Medium M was a slight modification of that described by Campbell *et al.* (1966). It had the following composition: 0.25 M-sucrose, 20 mM-tris-HCl, pH 7.6, 10 mM-magnesium acetate, 40 mM-NaCl, 100 mM-KCl, 10 mM- K_2HPO_4 , 1 mM-GTP, 6 mM-mercaptoethanol, 5 mM- α -oxoglutaric acid and 2 mM-ADP. Nine-tenths of this basic medium was mixed with one-tenth of the mitochondrial preparation, which had been resuspended in 0.3 M-sucrose-20 mM-tris-HCl, pH 7.6.

Medium B was similar to that described by Campbell *et al.* (1966) for their ribosomal preparation, and contained 0.25 M-sucrose, 20 mM-tris-HCl, pH 7.6, 10 mM-magnesium acetate, 40 mM-NaCl, 100 mM-KCl, 6 mM-mercaptoethanol, 0.05 μ M of each of the 20 amino acids except L-leucine, 0.1 mM-GTP, 2 mM-ATP, 5 mM-phosphoenolpyruvate, 10 μ g of pyruvate kinase/ml and 0.7-1 mg of pH5 enzyme/mg of protein of the microsomal fraction.

The pH of each medium was checked after mixing and the final pH of media A and M adjusted to 7.3 with KOH, whereas that of medium B was adjusted to pH 7.6.

Incubation conditions. Subcellular fractions were suspended in the appropriate medium and incubated in sterile stoppered vials with air as gas phase at 30°C in the presence of L-[U- ^{14}C]leucine or other radioactive amino acids. The vials were shaken at 100 cycles/min in a Dubnoff bath.

Measurements of radioactivity. At the completion of the incubation schedule vials were transferred to crushed ice and the contents diluted with the appropriate unlabelled amino acid (2 mg/ml) followed by cold trichloroacetic acid to a final concentration of 5% (w/v). The protein precipitate was washed by the method of Simkin & Work (1957). The precipitate was transferred to an Oxoid membrane by filtration, and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter with 10 ml of 4% (w/v) diphenyloxazole containing 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. The efficiency of counting was 90%. Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. Where incubations were carried out in the presence of albumin and cytochrome c, allowance was made for the presence of these proteins when calculating specific radioactivity.

Electron micrographs. These studies were done by a slight modification of the method of Hirsch & Fedorko (1968). The pellet or suspension of the subcellular fraction to be analysed was pre-fixed in a freshly made mixture of 0.8% glutaraldehyde in 0.1 M-sodium cacodylate buffer pH 7.4, and 0.6% osmium tetroxide in the same buffer. When the pre-fixation was made of the subcellular fraction in suspension, after 2 min in the cold the sample was centrifuged at 6000g for 1 min in an MSE centrifuge. The pellet was then transferred to fresh fixative and left on ice for 20 min. After the fixative had been decanted the pellet was washed twice with cold 0.9% NaCl and then resuspended in 0.25% uranyl acetate in 0.1 M-sodium acetate buffer, pH 6.3. After centrifugation the samples were left at 0°C for 20 min, and the pellet was trimmed into small blocks for dehydration and embedding. All samples were embedded in Epikote 812 (Epon) resin as described by Luft (1961). The sections were cut with glass

knives on an LKB Ultratome and post-stained with a saturated solution of uranyl acetate in 50% (v/v) ethanol and then with lead citrate (Venable & Coggeshall, 1965). The sections were examined in a Philips EM 300 microscope at 60 kV.

Bacterial contamination. All apparatus, media and instruments were sterile. Contamination at the end of the incubation was checked by placing a sample (0.1 ml) on to blood-agar plates which were kept at 37°C for 72 h. If more than 200 colonies/ml were found, the results were discarded.

RESULTS

Mitochondria-synaptosome mixture. The appearance of our standard preparation obtained from cortex homogenates by differential centrifuging in 0.3 M-sucrose-0.5 mM-EDTA, pH 7.3, is shown in Plates 1-3. Examination of Plate 1 shows that the preparation consists of a mixture of free mitochondria plus mitochondria sequestered within structures some of which are clearly synaptosomes. As shown in Plate 2 some of the structures contain polyribosome-like bodies, but rough endoplasmic reticulum is noticeably absent.

Incorporation of radioactive amino acids into the protein of the mitochondria-synaptosome suspension was dependent both quantitatively and qualitatively on the incubation conditions employed. In medium M, the rate of incorporation was about three times that in medium A. Thus, for example, a preparation from sucrose-EDTA that incorporated 1140 c.p.m./mg of protein in medium M incorporated only 495 c.p.m./mg in medium A. The kinetics of incorporation are shown in Fig. 1. The suspension in medium M incorporated amino acid linearly with time for only about 15 min whereas in medium A incorporation was almost linear for 30 min. The results given in Table 1 show that the difference in response in the two media is not simply a difference in efficiency, since in medium A protein synthesis is effectively inhibited by chloramphenicol and relatively insensitive to cycloheximide, whereas in medium M the same preparation is almost insensitive to chloramphenicol but strongly inhibited by cycloheximide. All preparations were strongly inhibited by puromycin and by azide, which blocks oxidative phosphorylation.

Several laboratories have now firmly established that in different types of eukaryotic cell, cycloheximide blocks synthesis in cytoplasmic ribosomes (cytoribosomes) whereas chloramphenicol blocks synthesis in mitochondrial ribosomes (mitoribosomes) (Clark-Walker & Linnane, 1966; Ashwell & Work, 1968, 1970a). Thus the results given in Table 1 indicate that the mitochondria-synaptosome fraction synthesizes protein by two distinct mechanisms, the balance between the two being

influenced substantially by the nature of the incubation medium. Table 1 shows that ribonuclease had a relatively feeble inhibitory action. This is probably because the synaptosomes are impermeable to ribonuclease.

Identification of the 80S ribosome as one of the active components in the mitochondria-synaptosome fraction. Since even traces of EDTA can promote degradation of cytoplasmic ribosomes it seemed advisable to use sucrose-tris to prepare the mitochondria-synaptosome fraction if 80S ribosomes were to be detected. This preparation had similar properties to that described in Table 1 and in medium M cycloheximide (100 $\mu\text{g}/\text{ml}$) produced over 80% inhibition of [^{14}C]leucine incorporation,

Fig. 1. Time-course of amino acid incorporation into the mitochondria-synaptosome fraction. The fraction (2 mg of protein/ml of incubation medium) was incubated with 0.3 μCi of [^{14}C]leucine (344 mCi/mmol) at 30°C as described in the Materials and Methods section; $\circ$, medium A; $\bullet$, medium M.

chloramphenicol (100 $\mu\text{g}/\text{ml}$) inhibited less than 15% and ribonuclease had scarcely any inhibitory action. Puromycin did of course inhibit protein synthesis almost completely.

The mitochondria-synaptosome preparation was pulse-labelled for 5 min at 30°C as described in Fig. 2, then sonicated for 1 min (Dawe Soniprobe set at 2mA) and lysed with a mixture of Brij-58 (0.1%) and sodium deoxycholate (0.25%). The product was layered on a sucrose gradient (for conditions see Fig. 2) and after centrifuging the gradient was separated into fractions and scanned for radioactivity. As Fig. 2 shows, there is a distinct peak of radioactivity at 80S and the formation of this radioactive peak is completely blocked by the presence of cycloheximide in the incubation mixture. It is notable, however, that in the presence of cycloheximide a small peak of radioactivity was detected at 55S, which we shall show to be due to mitochondrial ribosomes.

As was to be expected if the 80S peak is genuinely of ribosomes functioning in protein synthesis, the pulse label is completely discharged from this peak by exposure to puromycin for 1 min (see Fig. 2). In addition to the peak of radioactivity at 80S there is a second peak at about 100S. This could be polyribosome material, but we have not positively identified it as such. There was also a small pellet of heavy material at the bottom of the sucrose gradient. This material (probably polyribosomal) was seven times more radioactive than the original material and 70% of the radioactivity (c.p.m.) was discharged by incubation with puromycin for 1 min.

So far as we know this is the first report of the isolation of active cytoplasmic ribosomes in such preparations.

Protein synthesis by a brain cortex microsomal fraction and analysis of the ribosome profile of the microsomal fraction. As a check that the radioactive

Table 1. *Effects of inhibitors on incorporation of amino acids by the mitochondria-synaptosome fraction in media A and M*

The mitochondrial fraction (2 mg of protein) was suspended in 0.9 ml of the incubation medium + 0.1 ml solution of inhibitor and incubated with 0.2 μCi of [^{14}C]leucine (344 mCi/mmol) at 30°C for 15 min. For details of media see the Materials and Methods section.

Addition	Medium ...	Radioactivity			
		(c.p.m./mg of protein)		(percentage of control)	
		M	A	M	A
Water		1140	495	100	100
Cycloheximide (100 $\mu\text{g}/\text{ml}$)		162	362	14	73
Chloramphenicol (100 $\mu\text{g}/\text{ml}$)		1050	185	92	37
Puromycin (0.5 mM)		45	4	4	1
Azide (2 mM)		104	50	9	10
Ouabain (0.1 mM)		940	465	82	94
Ribonuclease (40 $\mu\text{g}/\text{ml}$)		890	565	78	114

EXPLANATION OF PLATE I

Thin section of a mitochondria-synaptosome fraction obtained in 0.3M-sucrose-0.5mM-EDTA, pH 7.3, showing a field containing synaptosomes (S). Within them it is possible to identify mitochondria (m) and synaptic vesicles (thin arrows). Ribosome-like structures (r) are seen in organelles which may be synaptosomes. There are some free mitochondria (M), empty membranous profiles resembling synaptosome 'ghost' (G), microsomes (mi) and some unidentified structures (X). The bar represents 1 μ m.

EXPLANATION OF PLATE 2

Thin section of the block used for Plate 1, showing at high magnification the occasional organelle which has not been unequivocally identified but which contains mitochondria (M) and, in close association with the mitochondria, a chain of ribosome-like particles (R) with sizes ranging between 160 and 200Å. The bar represents 0.5µm.

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EXPLANATION OF PLATE 3

Higher magnification of groups of ribosome-like particles (as on Plate 2) (arrowed), which are all of similar size, shape and density. The bar represents $0.2\mu\text{m}$.

EXPLANATION OF PLATE 4

Thin section of a purified mitochondrial fraction after incubation in medium A. Mitochondria profiles (M) are in a contracted state. Note the presence of membrane fragments (f) and the absence of well preserved synaptosomes (S). The bar represents 1 μ m.

Fig. 2. Detection of 80S and 55S ribosomes active in protein synthesis in a mitochondria-synaptosome fraction. Three identical batches were prepared as described in the text in the sucrose-tris medium. The organelle suspensions (10 mg of protein in each) were incubated each in 5 ml of medium M plus 2.0 μ Ci of L-[U- 14 C]leucine (344 mCi/mmol), 2.0 μ Ci of L-[U- 14 C]isoleucine (312 mCi/mmol) and 2.0 μ Ci of L-[U- 14 C]valine (260 mCi/mmol). Incubation was for 5 min at 30°C, after which the mixture was chilled to 0°C (except in the puromycin experiment) and centrifuged for 15 min at 12000g. Each pellet was resuspended in 0.2M-sucrose-10 mM-magnesium acetate-100 mM-KCl-10 mM-tris-HCl, pH 7.6, sonicated as described in the text and lysed by adding Brij-58 (0.1%) and sodium deoxycholate (0.25%), then layered over a linear (15-30%) sucrose gradient in 10 mM-magnesium acetate-100 mM-KCl-10 mM-tris-HCl, pH 7.6. The gradients were centrifuged at 27000 rev./min at 2°C for 3.25 h in the Spinco 30 rotor and 1 ml fractions were collected from the bottom of each gradient. The arrows indicate the position of the E_{260} markers, Krebs-cell 80S ribosomes (Kerr, Cohen & Work, 1966) and *E. coli* 50S ribosomal subparticles (Godson & Cox, 1970). ●, Control without added inhibitors; ▲, cycloheximide added at zero time (100 μ g/ml final concn.); ○, puromycin (200 μ M) added at 5 min and incubation continued for 1 min before chilling.

80S peak was due to the presence of active cytoplasmic ribosomes in the mitochondria-synaptosome preparation, we applied a similar pulse-labelling technique to the microsomal fraction from brain cortex. As shown in Fig. 3, cortex microsomal fractions were labelled and fractionated on sucrose gradients to give a radioactive 80S peak and the size of the peak was substantially increased by the sonication technique applied to our mitochondria-synaptosome preparation. Labelling of the 80S peak

with radioactive leucine was completely suppressed by cycloheximide or by ribonuclease but was unaffected by chloramphenicol (Table 2). A polyribosome peak is also evident in these experiments, but with an s value of 110S rather than the 100S reported in Fig. 2. We have not so far resolved this discrepancy but we think it may be related to the high lipoprotein content of our mitochondria-synaptosome preparations, since the sucrose gradients of this preparation showed a somewhat cloudy appearance in the region of the 110S peak.

Isolation of pure mitochondria from mitochondria-synaptosome preparations. The failure of ribonuclease to inhibit protein synthesis in the crude preparation, despite the fact that synthesis is mediated partly by 80S ribosomes sensitive to ribonuclease (Table 2), suggested that the boundary membrane was semi-permeable. Since mitochondrial protein synthesis is fairly resistant to osmotic shock (Haldar, Freeman & Work, 1967) it seemed likely that the structures containing mitochondria could be osmotically ruptured without serious damage to the synthetic capacity of the mitochondria. The mitochondria-synaptosome preparation was therefore subjected to mild osmotic shock (see the Materials and Method section) and the fraction sedimenting in 0.3M-sucrose-EDTA at 9000g in 10 min was further examined. Plate 4 is an electron micrograph of this fraction after incubation in medium A and it shows that the osmotic shock had ruptured the non-mitochondrial particles, so that the 9000g pellet now consisted largely of mitochondria. In addition there are some membrane fragments as well as partially disrupted synaptosomes.

Protein synthesis by purified brain-cortex mitochondria. When purified brain mitochondria were incubated under suitable conditions with [14 C]-leucine (Table 3), incorporation of label into mitochondrial protein was linear with time for 20 min (Fig. 4). By contrast with earlier results with the mitochondria-synaptosome fraction, protein synthesis in the purified mitochondria was completely resistant to cycloheximide and almost completely stopped by either chloramphenicol, puromycin or azide (Table 3). In these respects the preparation has all the characteristics expected if synthesis proceeds on a 55S mitoribosome (Ashwell & Work, 1970a). It is notable that when the osmotically purified mitochondria were incubated with an ATP-generating system instead of oxidizable substrate, protein synthesis did not follow a linear course but increased in rate after 10 min and continued for much longer than in conditions favouring oxidative phosphorylation (Fig. 4). Previous experiments with liver mitochondria (J. L. Coote & T. S. Work, unpublished work) had indicated that undamaged mitochondria used an

Fig. 3. Detection of 80S ribosomes and of polyribosomes active in protein synthesis in a brain-cortex microsomal preparation. (a) The microsomal fraction (15 mg of protein) isolated as described in the text was suspended in medium B (5 ml) and incubated at 30°C for 5 min with $3 \mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{leucine}$ (344 mCi/mmol). The chilled suspension was spun for 2 h at 100,000 g and the pellet was resuspended and treated with detergent as described in Fig. 2. (b) Microsomal fraction (15 mg) was divided in two and one fraction was sonicated as described in Fig. 2. The fractions were then layered on linear (15–30%) sucrose gradients in 5 mM-magnesium acetate–10 mM-KCl–10 mM-tris-HCl, pH 7.6, spun for 3.25 h and scanned for radioactivity as shown in Fig. 2. One gradient was also scanned for E_{260} . (a) ●, Radioactivity (c.p.m.); ○, E_{260} ; (b), radioactivity (c.p.m.) without sonication, ●; and after sonication, ○. The arrow indicates the position of the Krebs-cell 80S ribosomes used as marker.

Table 2. *Effect of metabolic inhibitors on brain microsomal protein synthesis*

Microsomal fraction (2 mg of protein) in 0.9 ml of incubation medium B + 0.1 ml of aqueous solution of the inhibitor was incubated with $0.3 \mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{leucine}$ (344 mCi/mmol) at 30°C for 15 min. Experimental details are given in the Materials and Methods section.

Addition	Radioactivity	
	(c.p.m./mg of protein)	(percentage of control)
Water	7000	100
Cycloheximide (100 $\mu\text{g}/\text{ml}$)	960	14
Chloramphenicol (100 $\mu\text{g}/\text{ml}$)	7250	103
Ribonuclease (40 $\mu\text{g}/\text{ml}$)	53	1

external ATP-generating system poorly but that $[\text{U-}^{14}\text{C}]\text{leucine}$ incorporation was linear for 1 h under conditions favouring oxidative phosphorylation. In contrast, osmotically damaged liver mitochondria behaved in a similar fashion to our purified prepara-

Table 3. *Effects of inhibitors on the incorporation of amino acids by the purified mitochondrial fraction*

The purified mitochondrial fraction (1 mg of protein) in 0.9 ml of medium A + 0.1 ml of inhibitor solution was incubated with $0.15 \mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{leucine}$ (344 mCi/mmol) at 30°C for 15 min. Experimental details are given in the Materials and Methods section.

Addition	Radioactivity	
	(c.p.m./mg of protein)	(percentage of control)
Water	1520	100
Cycloheximide (100 $\mu\text{g}/\text{ml}$)	1550	102
Chloramphenicol (100 $\mu\text{g}/\text{ml}$)	133	9
Puromycin (0.5 mM)	0	0
Azide (2 mM)	29	2
Ouabain (0.1 mM)	1520	100
Ribonuclease (40 $\mu\text{g}/\text{ml}$)	1430	94

tion of brain-cortex mitochondria. We suspected therefore that the purified brain mitochondria were somewhat damaged during incubation and this was

Fig. 4. Time-course of amino acid incorporation into a purified mitochondrial fraction. The mitochondria (1.5 mg of protein/ml) were incubated with 0.3 μ Ci of [14 C]leucine (344 mCi/mmol)/ml at 30°C as described in the Materials and Methods section; $\circ$, ATP-generating system; Δ , oxidizable substrate (Medium A).

confirmed by examination of an electron micrograph (results not shown) of cortex mitochondria after incubation in the ATP-generating system. By contrast with fresh liver mitochondria, the brain mitochondria isolated from the mitochondria-synaptosome fraction by osmotic shock were severely swollen and partly disintegrated after incubation in the ATP-generating system.

Identification of active mitochondrial ribosomes in purified brain mitochondria. Brain-cortex mitochondria were prepared as described above by mild osmotic shock of the mitochondria-synaptosome fraction. This preparation was pulse-labelled with [14 C]leucine (see Ashwell & Work, 1970a) either in medium A or in the ATP-generating system already described, and the mitochondria were sedimented at 9000g for 20 min. Each pellet was resuspended in a buffered sucrose-salt mixture and lysed with the neutral detergent Brij-58 (for details see Fig. 5). The lysate was layered on a linear buffered sucrose gradient and centrifuged for 3.25 h as described in Fig. 5. In each experiment the 50S ribosomal subparticle from *Escherichia coli* was added as an E_{260} marker. Fractions (1 ml) were collected from the gradient and scanned for radioactivity. As Fig. 5 shows there is a symmetrical peak of radioactivity sedimenting at 55S. This corresponds to the mitochondrial-ribosome peak previously identified by Ashwell & Work (1970a). Confirmation of the identity of this peak and of its function in mediating brain mitochondrial-protein synthesis is provided in Figs. 5, 6 and 7, where it is shown that labelling of the 55S peak is resistant to cycloheximide but is blocked almost completely by

Fig. 5. Detection of cycloheximide-resistant mitochondrial ribosomes in brain-cortex mitochondria. Mitochondria were isolated after osmotic shock as described in the text and added to two incubation vials (6 mg of protein and 5 ml of medium A in each). Then 3.0 μ Ci of L-[14 C]leucine (344 mCi/mmol) was added to both vials and cycloheximide (final concn. of 100 μ g/ml) to one. After incubation for 5 min at 30°C the suspensions were rapidly chilled to 0°C and centrifuged for 15 min at 10000g. The pellets were resuspended in 0.2 M-sucrose-10 mM-magnesium acetate-10 mM-KCl-10 mM-tris-HCl, pH 7.6, and Brij-58 was added, to a final concentration of 2 mg/mg of protein. The lysed mitochondria were layered over a linear (15-30%) sucrose gradient as described in Fig. 2. Tubes were centrifuged at 27000 rev./min for 3.25 h in the Spinco 30 rotor at 2°C. *E. coli* 50S ribosomal subparticles were included in all gradients as E_{260} markers (the peak indicated by an arrow). Fractions (1 ml) were collected from the gradient and each sample was monitored for E_{260} . The protein was precipitated with trichloroacetic acid, washed and its radioactivity counted as described in the text. $\bullet$, No cycloheximide; $\circ$, with cycloheximide.

chloramphenicol. As shown in Fig. 7, radioactivity is completely lost from the pulse-labelled mitochondrial ribosomes after exposure to puromycin for 1 min. When an ATP-generating system was used instead of medium A (cf. Figs. 8 and 9) a similar 55S peak was detected and the behaviour of the system towards chloramphenicol and cycloheximide was indistinguishable from that observed during oxidative phosphorylation. The main difference observed between the two types of experiment was that in the ATP-generating system treatment with puromycin for 1 min removed only 60% of the radioactivity. We interpret this difference as being due to the completion of a larger number of protein molecules in the ATP-generating

Fig. 6. Effect of chloramphenicol in blocking protein synthesis on mitochondrial ribosomes. Conditions were as described in Fig. 5. ●, No chloramphenicol; ○, with chloramphenicol (100 μ g/ml).

Fig. 7. Effect of puromycin in releasing label from mitochondrial ribosomes. Conditions were as described in Fig. 5 but in one tube puromycin (100 μ M) was added 1 min before the end of the incubation period. ●, No puromycin; ○, with puromycin.

system, but we have no proof that this is so. In the ATP-generating medium (Figs. 8 and 9) the mitochondria were preincubated with medium before

Fig. 8. Protein synthesis by brain-cortex mitochondria in an ATP-generating medium. Brain-cortex mitochondria isolated from the mitochondria-synaptosome fraction after osmotic shock (see the text) were suspended in the ATP-generating system (2 mg of protein/ml) and incubated for 20 min at 30°C. Radioactive leucine (0.6 μ Ci/ml, sp. radioactivity 312 mCi/mmol) was added and incubation continued for a further 5 min. Preparations were then chilled to 0°C and the mitochondria collected by centrifugation and lysed as described in Fig. 5. The radioactive mitochondrial ribosome peak was detected by scanning a sucrose gradient as described in the legend of Fig. 5. The *E. coli* 50S ribosomal subunit was used as E_{260} marker (the arrow indicates its position). ●, Control; ○, cycloheximide (100 μ g/ml); ▲, E_{260} (marker).

addition of labelled amino acid. This was done because, as shown in Fig. 4, in medium B there is a lag period before the mitochondria reach a steady state of protein synthesis.

DISCUSSION

Both Gordon & Deanin (1968) and Cunningham & Bridgers (1970) prepared brain-cortex mitochondria in what appeared to be a fairly pure state, but in each instance mitochondrial protein synthesis was strongly inhibited by cycloheximide. They both explain this observation by the assumption that the mitochondria of the synaptic knobs are specialized mitochondria with a special role in the synthesis of those proteins specifically required at the synaptic junction. Morgan & Austin (1968), Autilio *et al.* (1968) and Haldar (1970) favour the alternative hypothesis that two independent types of protein synthesis occur in the synaptosomal fraction, but the failure to find 80S ribosomes in synaptosomes or to prepare brain mitochondria resistant to inhibition of protein synthesis by cycloheximide has inevitably left the question completely open.

As shown in this paper, the washed mitochondria-synaptosome fraction contains 80S ribosomes as

Fig. 9. Effect of chloramphenicol (100 μ g/ml) in blocking protein synthesis on brain-cortex mitochondrial ribosomes. Conditions were as described in Fig. 8 except that in one incubation flask puromycin (100 μ M) was added 5 min after the radioactive leucine and incubation was continued for a further 1 min (cf. Fig. 7). ●, Control; ○, chloramphenicol (100 μ g/ml); ▲, puromycin added at 5 min and incubation then continued for 1 min before chilling.

well as 55S mitoribosomes, but by suitable conditions of osmotic shock this fraction can be converted to a preparation of mitochondria in which protein synthesis is completely resistant to cycloheximide and from which 55S mitochondrial ribosomes can be isolated. Protein synthesis on these mitochondrial ribosomes is inhibited completely by chloramphenicol. Part of the confusion in earlier work may have arisen from the use of sucrose in incubation media. As shown by Freeman & Haldar (1969), sucrose partially inhibits protein synthesis by mitochondria but it does not of course inhibit the operation of 80S ribosomes, so that, in experiments with cycloheximide, minor contamination of a mitochondrial preparation with cytoplasmic ribosomes will have a magnified effect if sucrose is used in the incubation medium. This effect is shown by the results in Table 1, where synthesis in sucrose-free medium is more strongly inhibited by chloramphenicol than synthesis in a sucrose-rich medium, and the reverse effect is observed with cycloheximide. We can explain the fact that protein synthesis by our preparation of cortex mitochondria was completely resistant to cycloheximide whereas that of Bosmann & Hemsworth (1970) was partially inhibited. In both cases osmotic shock was used as a means of releasing

mitochondria from the corresponding fraction of a homogenate, but whereas Bosmann & Hemsworth (1970) washed their preparation with 0.25M-sucrose, we included in the washing solution a low concentration of EDTA. This chelating agent in no way damages mitochondria, but it quickly renders 80S ribosomes inactive so that during the washing of an osmotically shocked homogenate EDTA will almost certainly eliminate any active 80S ribosomes that may still be partially protected by swollen but unruptured membranes (Whittaker, Michaelson & Kirkland, 1964). The possibility that brain mitochondria are heterogeneous remains open, since Neidle, Van den Berg & Grynbaum (1969) have shown that they are heterogeneous on a continuous sucrose gradient, but heterogeneity of this type is not exclusive to brain (Pollak & Munn, 1970) and the suggestion has been made that it reflects the physiological state of the mitochondria rather than any qualitative difference between them. The brain is the second animal tissue from which an active 55S mitochondrial ribosome has been isolated (see Ashwell & Work, 1970a; Coote, Rabbitts & Work, 1971). It seems likely therefore that in mammalian mitochondria protein synthesis always depends on the presence of a specific type of ribosome that differs in size from either the bacterial (70S) or the eukaryotic cytoplasmic ribosome (80S) but which nonetheless resembles the bacterial ribosome in its sensitivity to chloramphenicol and its resistant to cycloheximide.

One of the more revealing observations reported in this paper is the existence in the mitochondria-synaptosome fraction of 80S ribosomes that are highly sensitive to inhibition by cycloheximide yet are completely unaffected by ribonuclease or by chloramphenicol (as detected by the pulse-label technique). Further, the incorporation of amino acids into protein by the mitochondria-synaptosome fraction was inhibited by ouabain (Table 1) but this drug had no effect on the respiratory activity of purified mitochondria (cf. Blond & Whittam, 1964). It seems reasonable to attribute the cytoplasmic-type protein synthesis to structures containing cytoplasmic inclusions; synaptosomes are the main but not necessarily the only particles of this type in the mitochondria-synaptosome fraction. By electron microscopy we have detected, in this mitochondrial fraction, structures that contain ribosome-like particles. Also present in the mitochondria-synaptosome fraction there are other membranous particles (which are doubtfully synaptosomes) which contain polyribosome-like profiles (Plates 2 and 3). In view of the present results, and those of Morgan (1970) and Gambetti, Autilio-Gambetti & Gonatas (1970), we can state that 80S ribosomes exist in the mitochondrial fraction prepared from brain-cortex homogenates.

Although these 80 S ribosomes are not mitochondria-specific ribosomes, we cannot at present establish whether these ribosomes originate from synaptosomes or from other membranous particles present in the crude mitochondrial fraction.

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REFERENCES

- Ashwell, M. A. & Work, T. S. (1968). *Biochem. biophys. Res. Commun.* **32**, 1006.
- Ashwell, M. A. & Work, T. S. (1970a). *Biochem. biophys. Res. Commun.* **39**, 204.
- Ashwell, M. A. & Work, T. S. (1970b). *A. Rev. Biochem.* **39**, 251.
- Autilio, L. A., Appel, S. H., Pettis, P. & Gambetti, P. L. (1968). *Biochemistry, Easton*, **7**, 2615.
- Blond, D. M. & Whittam, R. (1964). *Biochem. J.* **92**, 158.
- Bosmann, H. B. & Hemsworth, B. A. (1970). *J. biol. Chem.* **245**, 363.
- Campbell, M. K., Mahler, H. R., Moore, W. J. & Tewari, S. (1966). *Biochemistry, Easton*, **5**, 1174.
- Clark-Walker, G. D. & Linnane, A. W. (1966). *Biochem. biophys. Res. Commun.* **25**, 8.
- Coote, J. L., Rabbitts, T. H. & Work, T. S. (1971). *Biochem. J.* **123**, 279.
- Cunningham, R. D. & Bridgers, W. F. (1970). *Biochem. biophys. Res. Commun.* **38**, 99.
- Freeman, K. B. & Haldar, D. (1969). *Biochem. J.* **111**, 653.
- Gambetti, P., Autilio-Gambetti, L., & Gonatas, N. K. (1970). *J. Cell Biol.* **174**, 68a.
- Godson, G. N. & Cox, R. A. (1970). *Biochim. biophys. Acta*, **204**, 489.
- Gordon, M. W. & Deanin, G. G. (1968). *J. biol. Chem.* **243**, 4222.
- Gray, E. G. & Guillery, R. W. (1966). *Int. Rev. Cytol.* **18**, 111.
- Haldar, D. (1970). *Biochem. biophys. Res. Commun.* **40**, 129.
- Haldar, D., Freeman, K. B. & Work, T. S. (1967). *Biochem. J.* **102**, 684.
- Hirsch, J. G. & Fedorko, M. E. (1968). *J. Cell Biol.* **38**, 615.
- Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 95.
- Kerr, I. M., Cohen, N. & Work, T. S. (1966). *Biochem. J.* **98**, 826.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Luft, J. H. (1961). *J. biophys. biochem. Cytol.* **9**, 409.
- Morgan, I. G. (1970). *FEBS Lett.* **10**, 273.
- Morgan, I. G. & Austin, L. (1968). *J. Neurochem.* **15**, 41.
- Neidle, A., Van den Berg, C. J. & Grynbaum, A. (1969). *J. Neurochem.* **16**, 225.
- Pollak, J. & Munn, E. A. (1970). *Biochem. J.* **117**, 913.
- Porterfield, J. S. (1960). *Bull. World Hlth Org.* **22**, 373.
- Roodyn, D. B., Reis, P. J. & Work, T. S. (1961). *Biochem. J.* **80**, 9.
- Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
- Venable, J. H. & Coggeshall, R. (1965). *J. Cell Biol.* **25**, 407.
- Whittaker, V. P. (1965). *Prog. Biophys. molec. Biol.* **15**, 39.
- Whittaker, V. P., Michaelson, I. & Kirkland, R. J. (1964). *Biochem. J.* **90**, 293.