The Effect of Convulsions Induced by Flurothyl on Ribonucleic Acid Synthesis in Rat Cerebral Cortex during the Recovery Phase

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1. The effect of convulsions, induced by flurothyl, on RNA synthesis in purified unfractionated nuclei and the cytoplasm of rat cerebral cortex was studied by using a double-label technique involving injection of $^{3}H$- and $^{14}C$-orotate intracisternally. Intact RNA was extracted in 80% yield by an enzymic method by using a proteinase in the presence of sodium dodecyl sulphate followed by deoxyribonuclease. Electrophoresis on 1.5% polyacrylamide-0.5% agarose gels revealed the presence of giant nuclear RNA of size up to approx. 300x10^6 daltons and mRNA of maximal mol.wt. 9x10^6-16x10^6.

2. Nuclear RNA synthesis was decreased to 27% in the first 15min after convulsions but rapidly increased, so that at 1 h it was 124% of the control, and at 6h 147%. Labelling of cytoplasmic RNA was decreased to 15% at 15min after convulsions but had not recovered to control values by 6h.

3. Analysis of radioactive gel patterns and the $^{3}H$/$^{14}C$ ratio at six time-points (15min–6h) showed that the major effect was inhibition of the processing of heterogeneous nuclear RNA resulting in a sharp decline in the export of newly synthesized RNA from the nucleus.

4. Cytoplasmic RNA patterns indicated that specific messengers were synthesized at different times during the recovery of the cell after convulsions.

Storage of information in the nervous system involves a sequence of macromolecular events which as yet are poorly understood. One plausible hypothesis is that nerve depolarization, with cyclic AMP as the possible mediator, triggers synthesis of certain RNA messengers from the DNA template in the cell nucleus of the neuron. This in turn leads to synthesis of proteins which are transported down the axon and leads to modification of the synaptic junction. One way of studying the problem is to disrupt the memory process by electroconvulsive shock or chemical convulsants [for a review, see Gibbs & Mark (1973)]. A second reason for choosing convulsants is that seizure therapy is commonly used to treat endogenous depression in psychiatric patients. Many aspects of this, including the biochemical consequences, are unclear.

The convulsant used in this study with female rats was flurothyl [bis-(2,2,2-trifluoroethyl) ether, $\text{F}_3\text{C}-\text{CH}_2\text{-O-CH}_2\text{-CF}_3$], an inert volatile liquid. One of its advantages is the ease of application and its elimination from the lungs as an unchanged vapour. In humans, this occurs with a half-life of 30min. Flurothyl produced retrograde amnesia in mice as long as 6h after the learning trial (Bohdanecky et al., 1968). Small & Small (1972) have shown flurothyl to have similar effects to electroconvulsive shock in humans, although the mechanism of inducing convulsions may be very different.

Much attention has been focused on RNA since Hyden first proposed a theory of memory. A decrease in the total RNA content has been claimed for the cerebral cortex of cats, 1min after electroshock (Mihailovic et al., 1958), in the crude nuclear fraction of rat brain, during the convulsive phase (Chitre et al., 1964) and in subcellular fractions of the cerebral cortex of adult mice measured 20min after convulsions (Essman, 1972). However, no significant changes in RNA content were observed 1h after convulsions (Brill, 1967) and a return to normal content was found during the post-convulsive phase (Chitre et al., 1964). After acute metrazol-induced seizures, Pevzner & Saudargene (1971) found a 30% decrease in the RNA content of motor neurons of the spinal cord and the sensory neurons of spinal ganglia, lasting up to 3h. The neurogial RNA content had also decreased initially in both cases, but at 3h they had returned to normal for glia in the spinal cord whereas they were still decreased at 18h for the glia in the spinal ganglia. It is not surprising that Engel & Morrell (1970) found no significant differences in RNA content between neuronal populations of mirror epileptic foci of rabbit cortex.

Experiments in vitro with electrical stimulation of cortex slices showed that $^{3}H$uridine incorporation into RNA was decreased after 30min incubation (Prives & Quastel, 1969). Orrego (1967) in similar experiments had reported a 40% decrease. But quite
contrary results were obtained by Bharucha & Elliott (1974). Rats underwent electroconvulsive shock and were killed during convulsions or 30min later. In both cases, after 1h incubation of cortex slices in [3H]uridine there was increased synthesis of RNA. Thus studies so far indicate that changes in RNA content depend on the exact nature and strength of the stimulus, the time examined after seizures and the cell type. As yet no examination of the class of RNA molecules synthesized after convulsions has been attempted.

In such a complex process there are many factors to be examined. In the present work, the effect on RNA synthesis in the cerebral cortex of rats during the recovery phase after convulsions induced with flurothyl has been studied. The incorporation in vivo of orotic acid into RNA of unfractionated nuclei and cytoplasm was measured with a double-label technique at various times after convulsions. The RNA was subsequently fractionated on polyacrylamide-agarose gels. A method for isolating intact RNA was modified from that used successfully with single nuclei by Ringborg & Rylander (1971). This revealed the presence in brain of giant HnRNA* with very large molecular weights.

Materials and Methods

Chemicals

Halothane was obtained from I.C.I., Alderley Park, Cheshire, U.K.; Simplex Rapid Acrylic Denture Repair Material and Cold Curing Acrylic were from Dental Fillings, Grayling Road, London N.16, U.K. Flurothyl (trade name, Indolcon; Ohio Chemical and Surgical Equipment Co., Madison 10, Wis., U.S.A.) was a generous gift of Dr. O. Pratt, The National Hospital for Nervous Diseases, Queens Square, London W.C.1, U.K. Proteinase from Streptomyces griseus type VI, DNAase from bovine pancreas (electrophoretically purified) and RNAase type IIA from bovine pancreas (proteinase free) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; Soluene-350 came from Packard Instruments, Caversham, Berks., U.K.; hepamin was from Evans Medical, Speke, Liverpool, U.K.; actinomycin D was from Merck, Sharp and Dohme, Hoddesdon, Herts., U.K. RNAase-free sucrose from Cambrian Chemicals, Croydon, Surrey, U.K., was used throughout. Formamide, dimethyl sulfoxide, acrylamide and bisacrylamide, purchased from BDH Chemicals, Poole, Dorset, U.K., were all recrystallized or distilled. [5-3H]Orotic acid (specific radioactivity 17.9Ci/mmole) and [6-14C]orotic acid (specific radioactivity 61mCi/mmole) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of the highest purity commercially available.

Injections of animals

Female albino rats (Sprague–Dawley strain), 4–5 weeks old and weighing 90g, were used in all experiments. The rats were pre-medicated with atropine (0.05mg/kg) 5min before anaesthesia. Animals were anaesthetized in 45s by bubbling O₂+CO₂ (95:5) through halothane into a large desiccator. Anaesthetized animals were injected with radioactive material intracranially as described by Austoker et al. (1973). The hole was immediately sealed with dental cement. Rats recovered normal posture within 10s to 2min after injection. In order not to increase the hydrostatic pressure of the blood and cerebrospinal fluid, the volume of the injection was 40μl. An appropriate volume of the solution of [5-3H]orotic acid was freeze-dried 2h before injections. [3H]- or [14C]-orotic acid was dissolved in sterile 0.9% NaCl containing 1.36μmol of NaOH in a total volume of 30μl. This was taken up into a Hamilton Microsyringe already containing 10μl of 0.9% NaCl to decrease losses from leakage at the site of injection. For each time-point investigated, three control rats were injected with [5-3H]orotic acid (130–160μCi) and three rats which were to undergo convulsions were injected with [6-14C]orotic acid (28–45μCi).

Of the radioactivity injected, 5–7% was taken up by the cerebral cortex. Total radioactivity found in the homogenates of the pooled brains ranged from 56×10⁶ to 94×10⁶ d.p.m. for [3H]orotic acid and 9×10⁶ to 19×10⁶ d.p.m. for [14C]orotic acid. Larger amounts of radioactivity were injected at the earlier time-points in order to increase the labelling of RNA, especially that of convulsed rats.

Convulsive treatment

Flurothyl (0.2ml), the convulsive agent, was absorbed on a cottonwool pad attached to the top of a chromatography tank (20cm×20cm×8cm) with a lid. It was allowed to evaporate for 3min. At 2min after recovery from halothane anaesthesia, the rats were placed in the tank. Tonic-clonic convulsions began within 15–60s. Animals were removed immediately full seizure had been elicited. Zero time was taken from the moment of injection of label. Rats underwent convulsions 4–5min after injection. No obvious differences in behaviour were observed between control and convulsed rats at 5min after recovery from seizures. Vernadakis & Woodbury (1969) have shown that the rat brain has sufficiently matured by 16 days to undergo convulsions. However, on some occasions slight overexposure to the
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Drugs produced massive respiratory secretions which were lethal. Since rats can be exposed to flurothyl for 6min before death occurs from anoxia (Truitt & Ebersharger, 1960), the respiratory secretions were probably due to an interaction of flurothyl and halothane. Possibly an increased dose of atropine would eliminate this problem.

At various times after injection of labelled orotic acid, rats were killed by cervical dislocation and decapitation. The cerebrum was removed and the cerebral cortex was dissected out on wet filter paper in a Petri dish cooled in ice. The cortex was immediately frozen in liquid N₂. The whole operation took 4min.

Isolation of nuclear and cytoplasmic fractions

The three brains from the convulsed rats and the three from the controls for each time-point were pooled and thawed in 40ml of cold 0.32M-sucrose, 1 mM-sodium cacodylate, 3 mM-MgCl₂, pH6.5, containing 1.0mg of the sodium salt of naphthalene-1,5-disulphonic acid/ml, to inhibit RNAase, and 10μg of actinomycin D/ml. The latter was necessary to prevent further synthesis of all RNA species, as this can continue in isolated nuclei. The brains were homogenized by hand with 25 up-and-down strokes in a Potter-Elvehjem homogenizer with 250μm clearance. The homogenate was filtered through nylon bolting cloth and washed with a further 10ml of sucrose solution. The filtrate was centrifuged in polypropylene tubes with fitted caps at 900g for 10min. The postnuclear supernatant was collected.

The nuclear pellet was washed with 30ml of 0.32M-sucrose, 1 mM-MgCl₂, 1 mM-cacodylate, pH6.5, and 1.0mg of naphthalene disulphonate/ml (solution A) and re-centrifuged. The crude nuclear pellet was resuspended in 40ml of solution containing 1 mM-MgCl₂, 1 mM-sodium cacodylate, pH6.5, and 1 mg of naphthalenedisulphonate/ml, and the final concentration of sucrose was adjusted to 50% (w/w) with the aid of a refractometer. The mixture was centrifuged at 5°C in the MSE 6×15 rotor at 76000g for 60min (rav. = 10.97cm). The purified nuclear pellet was suspended in 3.0ml of solution A. Numbers of nuclei were measured by counting a sample, diluted with 0.9% NaCl in a Coulter counter.

Extraction of RNA from nuclei

All glassware, centrifuge tubes, pipettes, Eppendorf tubes and tips were washed with chromic acid or treated with 0.1% diethyl pyrocarbonate to ensure minimal degradation by extraneous RNAase. All solutions, including sucrose, were filtered and sterilized. Gloves were worn throughout the operations.

Proteinase to a concentration of 2.5mg/ml was dissolved in 0.04M-Tris-HCl, pH7.4, containing 2% (w/v) sodium dodecyl sulphate. To digest any traces of RNAase, the proteinase solution was pre-digested at 37°C for 90min, after which it was centrifuged at 2000g for 15min to remove a small sediment. Sufficient proteinase solution was added to the nuclear suspension in solution A so that the proteinase/protein ratio was 1:1 (w/w), assuming 30μg of protein/nucleus. The final incubation mixture contained 1% sodium dodecyl sulphate, 1.25mg of proteinase/ml, 0.02M-Tris-HCl, pH7.4, and 3×10⁸-4.5×10⁹ nuclei. The nuclei were digested at 37°C for 90min with shaking. Then 2 vol. of ethanol containing 2% (w/v) potassium acetate was added and the mixture left overnight at -20°C. The remaining actinomycin D that had been bound to the nuclei was removed at this stage. The precipitate of RNA and DNA was recovered by centrifugation at 1500g for 10min. It was washed with 2×5ml of 70% (v/v) ethanol. All washes were done with solutions cooled to 4°C. To remove entrapped proteinase, the pellet was resuspended in 1% sodium dodecyl sulphate (2ml). This was left at 20°C for 15-30min with gentle shaking until the compact DNA pellet had a much looser structure. It was then precipitated as described above. After 2-4h at -20°C, the pellet was collected and washed with 2×5ml of 70% ethanol and once with 5ml of ethanol. Removal of DNA was done by suspending the pellet in a final volume of 2ml containing 0.02M-Tris-HCl buffer, pH7.8, 10mM-MgCl₂, 0.1M-NaCl and DNAase in the ratio 1 μg to 10 μg of DNA. To minimize the effect of any trace of RNAase, heparin was included at a final concentration of 100μg/ml. The suspension was left at 0°C with frequent agitation for 2-3h, during which clumps of DNA dispersed. After an additional 30min, 500μg of proteinase in 1% sodium dodecyl sulphate (0.2ml) was added to remove any traces of protein. The RNA was precipitated, after 10min, by the addition of ethanol and potassium acetate (2%, w/v). After 2h at -20°C, the RNA was collected by centrifugation and washed twice with 70% ethanol.

Lipid impurities were then extracted by washing the pellet once with 5ml of chloroform-methanol (2:1, v/v) and once with 5ml of chloroform-methanol-water (33:67:2, by vol.) for 5min at 0-4°C with frequent vortex-mixing. Failure to remove the lipids resulted in the formation of large micelles, which prevented RNA from entering the gels during electrophoresis. The precipitate was washed once with ethanol. To remove entrapped oligodeoxyribonucleotides, the RNA was dissolved in 1% sodium dodecyl sulphate (2ml). It was re-precipitated and washed with 70% ethanol and ethanol as described above. The purified RNA pellet was dried under vacuum for 10min. It was dissolved in 200-400μl of gel buffer containing 1% sodium dodecyl
sulphate, 18% (v/v) glycerol and 0.002% (w/v) Bromophenol Blue as a marker. The solution was frozen in liquid N₂ and stored at -20°C. Whenever the RNA samples were removed or placed in the freezer, they were thawed rapidly and frozen rapidly to prevent any deterioration of the RNA which might occur if the sodium dodecyl sulphate came out of solution.

Cytoplasmic RNA

The postnuclear supernatant was made 1% (w/v) with respect to sodium dodecyl sulphate. Proteins and nucleic acids were precipitated by the addition of 2vol. of ethanol and stored at -20°C overnight. The gel profiles of cytoplasmic RNA are identical if the time of storage is extended to 4 days, or if the digestion with proteinase is undertaken immediately and without prior precipitation of the RNA indicating that the storage at -20°C before digestion of protein is not harmful to the RNA. The precipitate was collected by centrifugation at 1500g for 10 min and washed twice with 70% ethanol, once with ethanol and then dried under vacuum. It was incubated with proteinase solution by using 5mg of proteinase/g of original tissue for 90 min at 37°C. The subsequent procedures were identical with those described above for nuclei, except that the DNAase step was omitted. Two resuspensions in 1% sodium dodecyl sulphate (5ml each) and re-precipitations were done before final suspension in gel buffer (0.6-1.0ml).

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

Polyacrylamide gels (1.5 and 2.4%) containing 0.5% agarose were prepared as described by Peacock & Dingman (1968). The acrylamide and agarose solutions were mixed at 37-40°C, left at 37°C for 30 min to polymerize partially, and then overnight at 20°C. The ends of the gels were levelled with a sharp scalpel blade. The gel buffer consisted of 40mm-Tris, 20mm-sodium acetate and 2mm-EDTA, adjusted to pH 7.8 with acetic acid. It was made 0.5% with respect to sodium dodecyl sulphate. Gels were pre-run for 1 h at 5mA/tube. The RNA samples were loaded in volumes of 10-100μl containing 20-130μg of RNA and slowly electrophoresed into the gel pores at 1.25mA/tube for 1 h at constant current. Electrophoresis was continued for 2 h at 5mA/tube. The gels were gently removed from the Perspex tubes and transferred to fixative (water–methanol–acetic acid, 6:3:1, by vol.) for 1 h with occasional mixing. The gels were scanned in a Joyce–Loebl u.v. scanner at 265 nm. If the gels were to be stained, they were placed in 50ml of 0.4M-sodium acetate–0.4M-acetic acid–0.05% Methylene Blue for 1 h and destained with water for 48 h.

DNAase treatment

RNAase was boiled for 15 min at 100°C to destroy any contaminating enzymes. Nuclear RNA (90 μg) was incubated with RNAase (14 μg) in a total volume of 110μl containing 0.5% sodium dodecyl sulphate and heparin (140 μg) for 11 h at 37°C.

Slicing of gels for radioactivity determinations

Immediately after scanning, the gels were sliced in an Aliquogel Fractionator [Gilson Medical Electronics (France), Villiers-le-Bel, France] into 1 or 2mm fractions (Gilson et al., 1972). Slices were pumped directly into scintillation vials with 0.35ml of Soluene-350. Difficulties arose in the initiation of slicing. A dyed spacer gel of the same concentration was placed on top of the radioactive gel and collection was begun after an appropriate number of slices. However, the first slice of the radioactive gel was always a fraction of 1 or 2mm. Thus in duplicate gels, the labelling pattern showed larger or smaller peaks, especially with a heterogeneous pattern, depending on the exact starting point of the slicing.

Scintillant (0.5% PPO (2,5-diphenyloxazole) and, 0.025% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in toluene) was added directly to the vials, which were left overnight at 20°C before cooling and counting for radioactivity on an Intertechnique liquid-scintillation spectrometer (ABAC SL40). It was found that vials could be left at 4°C for a week with no loss of efficiency of counting. The efficiency of 1H counting was 35.0%, and 14C above 3H, 55%. The spill-over of 14C into the 3H channel was 12%. Radioactivity of samples was counted twice for 10 min and the average d.p.m. computed from quench curves stored in the computer of the counter. Test electrophoretic runs under denaturing conditions [50% (v/v) formamide] of HeLa-cell 28S rRNA, labelled with 3H, showed that recovery of radioactivity (d.p.m.) from the gel was 92–96%. After overnight fixation, there was no preferential loss of labelled 7S rRNA. A 1.5% polyacrylamide-0.5% agarose gel was made containing known amounts of [3H] and [14C]orotic acid in the ratio 2.53:1. The recovery from 1mm slices was 98.6 and 98.5% respectively, with a mean error in d.p.m. of ±2.0% and ±2.24%. The recovery from 2mm slices was 94.6 and 97.4% for 3H and 14C respectively, with a mean error after slicing and counting of ±2.12% and ±3.39%. For 1mm slices the mean error of the 3H/14C ratio was ±2.74% and for 2mm slices ±1.65%. The errors were calculated for a pre-set count of 10000 c.p.m. for 3H or 14C.

Measurement of RNA and DNA

RNA was measured as described by Balazs et al. (1968), except that alkaline hydrolysis was for 1 h with
EXPLANATION OF PLATE 1

Separation of nuclear and cytoplasmic rat brain RNA by gel electrophoresis

Nuclear RNA (41 μg in 25 μl) was electrophoresed on 1.5% polyacrylamide–0.5% agarose gels for 2h (gels 1 and 2). They were fixed and stained with Methylene Blue as described in the Materials and Methods section. The approximate s values are indicated for the bands of gel 1. Gel 3 was nuclear RNA treated with RNAase for 11 h before electrophoresis. The staining of cytoplasmic RNA (33 μg in 20 μl) after electrophoresis on 1.5% polyacrylamide and 0.5% agarose gels for 2h is shown in gel 4 and the same material (33 μg in 10 μl) on 2.4% polyacrylamide–0.5% agarose in gel 5.
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1M-KOH. RNA concentration was calculated from a standard hydrolysed under the same conditions (E50/50 = 300). All operations were done in I ml Eppendorf tubes so that only small amounts of sample were necessary. DNA was extracted twice at 80°C with 0.5M-HClO4. The concentration was calculated from the extinction at 270nm, compared with a DNA standard (E1% = 195) extracted under the same conditions. To calculate the amount of DNA in the original homogenates, a correction was made for material absorbing in the u.v., but which was not DNA. This was done by extracting a portion of the cytoplasmic fraction under similar conditions.

Results

Isolation of nuclear RNA

From measurements of DNA, it was estimated that the recovery of purified nuclei from homogenates of rat cerebral cortex was 50%. The isolated nuclei contained 200–250μg of RNA/g wet wt. of tissue, representing 9–12% of the RNA concentration found in the homogenate. The extraction of RNA from nuclei was performed by first digesting the nuclei with the proteinase from Streptomyces griseus. This enzyme has a wide specificity and attacks virtually all peptide bonds. The digestion is carried out in the presence of 1% sodium dodecyl sulphate to loosen the attachment of protein to nucleic acids. Extension of the period of digestion from 90 min to 6 h did not alter the gel profiles of the nuclear RNA. DNA was removed by digestion with DNase. This step was followed by a second digestion with proteinase. The recovery of nuclear RNA, determined by measurements of radioactivity in a form that is insoluble in acid, but degraded by alkali and RNAase, from nuclei that were labelled for 1 h with [3H]uridine in vivo is of the order of 95%. The E260/E280 ratio of the nuclear RNA was 1.98 and the E260/E230 ratio was 2.16. The re-precipitation of the RNA by ethanol in the presence of sodium dodecyl sulphate is effective in removing traces of residual proteins, and gels of the RNA do not take up protein stains, apart from weak staining at the positions of the two components present in the proteinase. Two additional precipitations will remove these traces of proteinase.

Purified RNA was electrophoresed in gels containing 1.5% polyacrylamide and 0.5% agarose in the presence of 0.5% sodium dodecyl sulphate. Plate 1 shows nRNA after fixation and staining with Methylene Blue (gels 1 and 2). Stain was clearly visible within 0.2 cm from the top of the gel. That this was due entirely to staining of RNA is shown by gel 3, which displays the pattern of nRNA treated with RNAase, as described in the Materials and Methods section. The distribution of labelled nRNA in the gel confirmed the presence of nRNA within the first few millimetres of the top of the gel (see later results). Estimates of the molecular weight and s value of the RNA in this region may be made by extrapolation of the plot of the distance of migration of 28S, 18S and 4S RNA against the logarithms of molecular weights and s values. For six separate preparations, values within the range 300–400S (mean 330S) were obtained. If the extrapolation to an s value of this order is valid, and on the assumption that the molecular weights of 28S, 18S and 4S RNA are 1.76×106, 0.67×106 and 2.5×104 respectively (Wellauer & Dawid, 1974), the molecular weight of the largest heterogeneous nuclear RNA molecules would be at least 3×106.

A profile of nRNA, scanned at 265nm is shown in Fig. 1. The HnRNA appeared as a broad zone with two subsidiary peaks at 125S and 57S. There was also a small peak at 39S, which is probably a precursor of rRNA (pre-rRNA). The exact shape of the profile for HnRNA varied slightly. Sometimes the peak at 125S degenerated into a shoulder. Estimates from six preparations gave a range of 98–125S for the first visible peak of HnRNA, 53–60S for the second and 37–40S for putative pre-rRNA. Low-molecular-weight nuclear RNA showed a sharp peak as the 5S position and on either side two diffuse bands whose amounts were variable (Fig. 1). These bands were obscured after staining by heparin added during the treatment with DNAsase (Plate 1, gels 1 and 2). Heparin does not absorb in the u.v., but stains with Methylene Blue.

Estimates of the proportions of the major components of nRNA were made for five separate samples by determination of the areas under the curve of the u.v. scans of the gels after they had been fixed overnight to remove oligodeoxyribonucleotides and other gel artifacts. The baseline was taken as the lowest point on the u.v. scan and verified by the scanning of blank gels. HnRNA plus pre-rRNA comprised an average of 38% (range 30–50%) of the total nRNA.

Several lines of evidence support the view that the heterogeneous nuclear RNA seen in Plate 1 and Fig. 1 is not an artifact of the method of isolation. First, if 28S rRNA from HeLa cells, purified by extraction with phenol, labelled with [3H]uridine and isolated by density-gradient centrifugation, is added to the preparation of RNA from brain before the treatment with DNAsase, the gel profile shows a sharp peak of radioactivity at a position identical with that of HeLa-cell 28S rRNA and no radioactivity in the region of the gel above 28S rRNA. Thus there is nothing in our preparations of nuclear RNA that causes aggregation or smearing of RNA during electrophoresis. It should be emphasized that phenol can cause aggregation (MacNaughton et al., 1974; Rhoads et al., 1973). Secondly, only very small amounts of material are seen above 28S RNA in the preparations of cytoplasmic RNA (gel 4, Plate 1).
After electrophoresis, 1.5% acrylamide gels were fixed overnight in water–methanol–acetic acid (6:3:1, by vol.) and scanned at 265 nm. (a) nuclear RNA (54 µg in 30 µl); (b) cytoplasmic RNA (31 µg in 20 µl). A logarithmic plot of the molecular weights versus migration distance is shown for nuclear RNA. The position of 4S RNA was calculated from the migration distance of cytoplasmic 4S RNA electrophoresed at the same time. Approximate s values are indicated.
Thirdly, treatment of the RNA with dimethyl sulfoxide had only a minor effect on the gel pattern. One volume of nRNA in electrophoresis buffer was incubated with an equal volume of dimethyl sulfoxide at 20°C for 1 h before electrophoresis. Under these conditions, quantitative release of 7 S rRNA from 28 S rRNA occurred, and essentially all internal breaks in preparations of rRNA are revealed with the appearance of fragments of rRNA of lower molecular weight. The treatment with dimethyl sulfoxide causes only a minor shift of the staining near the top of the gels in the profiles of the brain nRNA and of the distribution of radioactivity such as that shown in Fig. 5(b). This slight displacement towards a lower molecular weight may be due to some internal single-strand breaks that occur during isolation of the RNA or during processing in vivo. Increasing the time of treatment with dimethyl sulfoxide to 18 h, or substitution of 80% (v/v) formamide, has effects identical with those of 50% dimethyl sulfoxide for 1 h.

Isolation of cytoplasmic RNA

The RNA measured in the postnuclear supernatant was equivalent to a concentration of approx. 900 μg/g wet wt. of tissue. This represented 37-46% of the total RNA in the original homogenate, the rest of the RNA being lost in the nuclear wash and the material not sedimenting in 50% (w/w) sucrose. Purified RNA could be isolated from the postnuclear supernatant in yields of 40-55%. The low yield was due to losses during the washes with ethanol. This affected cytoplasmic RNA far more than nuclear RNA. It was later found that yields could be increased to 80% if longer intervals were allowed between resuspension in ethanol and centrifugation.

The E_{260}/E_{320} ratio was 1.91 and the E_{260}/E_{230} ratio was 1.20. The low value of the latter was due to significant amounts of glycopeptide impurities. Electrophoresis of the cytoplasmic RNA on 1.5% polyacrylamide-0.5% agarose gels revealed no components that stained with Methylene Blue with values greater than 28 S (gel 4, Plate 1). On 2.4% polyacrylamide gels, the minor bands of cytoplasmic RNA were well resolved (gel 5, plate 1). Other workers, who used phenol in the extraction, have reported the presence of minor components in brain cytoplasmic RNA (Campagnoni et al., 1971). Two faint bands moving more slowly than 28 S were visible in this gel, which were found to be DNA. The faint band migrating ahead of 4 S RNA, seen in gel 4 (Plate 1), was caused by glycopeptides which co-precipitated with RNA from 70% (v/v) ethanol but did not interfere in the electrophoresis. This was demonstrated by staining with Schiff's reagent after periodate oxidation (Segrest & Jackson, 1972). The low-molecular-weight glycopeptides slowly diffused out of the gel during fixation. Glycopeptides could not be detected by Schiff's reagent in nRNA preparations. Small amounts may have been present but were obscured by the background of staining caused by the agarose which is a component of the gels. The absence of RNA greater than 28 S from the preparations of cytoplasmic RNA was confirmed by scanning the gels in u.v. light (Fig. 1b). Even when 120 μg of RNA was loaded on a gel, no species moving more slowly than 28 S was observed.

**Effect of the convulsive agent flurothyl on RNA synthesis during the recovery phase**

To allow a direct comparison of the synthesis of RNA in untreated rats with that of rats recovering from convulsions induced by flurothyl, the RNA from normal rats was labelled with [3H]orotic acid and the treated animals were injected with [14C]orotic acid. The RNA was extracted from the pooled cerebral cortices to eliminate any differences due to random RNAase activity. The experimental plan based on both the use of two different radioactive isotopes to
The specific radioactivity of nuclear RNA (α) and cytoplasmic RNA (β) was calculated from the concentration of RNA by using $\varepsilon_{260}^\alpha = 240$. The amount of radioactivity was corrected for the total d.p.m. of $[^3H]$- and $[^14C]$-orotate injected, as measured by the radioactivity of the homogenate. For comparison of experiments, the radioactivity of the homogenate was arbitrarily fixed at $7.5 \times 10^7$ d.p.m. for $[^3H]$orotic acid and $1.5 \times 10^7$ d.p.m. for $[^14C]$orotic acid. The actual values were close to these numbers.

$$\text{Relative specific radioactivity of }[^3H]\text{-labelled RNA} = \frac{\text{d.p.m./µg of RNA} \times 7.5 \times 10^7}{\text{total }[^3H] \text{ d.p.m. of homogenate}}$$

Similar corrections for $[^14C]$ radioactivity were calculated. The specific radioactivity of control rats is shown by ○ and of convulsed rats by Δ.

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Fig. 3. Change with time in the specific radioactivity of nuclear and cytoplasmic RNA for normal and convulsed rats

The specific radioactivity of nuclear RNA (α) and cytoplasmic RNA (β) was calculated from the concentration of RNA by using $\varepsilon_{260}^\alpha = 240$. The amount of radioactivity was corrected for the total d.p.m. of $[^3H]$- and $[^14C]$-orotate injected, as measured by the radioactivity of the homogenate. For comparison of experiments, the radioactivity of the homogenate was arbitrarily fixed at $7.5 \times 10^7$ d.p.m. for $[^3H]$orotic acid and $1.5 \times 10^7$ d.p.m. for $[^14C]$orotic acid. The actual values were close to these numbers.

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Similar corrections for $[^14C]$ radioactivity were calculated. The specific radioactivity of control rats is shown by ○ and of convulsed rats by Δ.

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label the RNA from normal and treated animals and the processing of the brains from the two groups of animals together, enables a determination of the effect of the convulsive agent on RNA synthesis to be made even if some small but variable degree of breakdown of RNA occurs during its isolation or if the resolution of RNA species on gels varies slightly from one experiment to another.

The rate of labelling of nuclear and cytoplasmic RNA from convulsed rats was expressed as a percentage of the control at various times after convulsions (Fig. 2). The results were normalized with reference to the radioactivity of the homogenate. This eliminated differences between experiments caused by route of injection, amounts of radioisotopes injected and rate of uptake of label. At 15min, the rate of nRNA synthesis was drastically decreased to 27% of the control. The rate rapidly increased, until at 90min RNA synthesis in the nucleus was greater in convulsed rats than the control (124%). This had increased to 147% by 6h. An attempt was made to assess the effects of convulsions on the size of the acid-soluble nucleotide pools labelled from the precursor orotic acid by using the method of Marchisio & Bondy (1974) which involves adsorption of the nucleotides to charcoal. Unfortunately the unchanged orotic acid also bound to charcoal and vitiated this procedure.

The rate of labelling of cytoplasmic RNA was similarly decreased at 15min to 15% of the control (Fig. 2). By 90min, this had only reached 64% of the control value. The small decrease to 55% in the rate of labelling at 2h may be attributed to slight variations in the reaction of rats to convulsive treatment. However, in contrast with nRNA, the rate of labelling of cytoplasmic RNA had not reached control values even after 6h (87%). As shown in the following experiments, most of the labelling of cytoplasmic RNA, especially at 15min, was due to tRNA.

The specific radioactivity of the nRNA of convulsed rats showed a rapid increase, reaching a maximum at 2h, but had declined by 6h (Fig. 3a). The maximum specific radioactivity was greater for treated rats and correlated with the increased rates of synthesis. There was a small error in the estimates of the concentration of RNA because of variable amounts of oligodeoxyribonucleotides remaining after DNAase treatment and precipitable in 70% ethanol. Later it was found that these could be eliminated by mechanical dispersion of the DNA.
pellet at the beginning of the DNAase treatment. Saborio & Aleman (1970) and Balazs & Cocks (1967) have both shown that the specific radioactivity of nRNA was maximal at 3h.

The rate of incorporation of labelled precursor into cytoplasmic RNA was biphasic, showing a slow increase until 1½h and a more rapid increase at 2h (Fig. 3b). By 6h, the specific radioactivity of cytoplasmic RNA was five times that at 1½h for control rats and almost seven times for convulsed rats.

**Labelling pattern of brain nuclear RNA from normal rats and those recovering from convulsive seizures**

Double-labelled nRNA was fractionated on 1.5% polyacrylamide–0.5% agarose gels. After 1 h fixation,

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**Fig. 4. Labelling pattern of nuclear RNA from normal and convulsed rats at various times after injection**

Double-labelled nuclear RNA was electrophoresed on 1.5% polyacrylamide–0.5% agarose gels for 2h. The gel was fixed, scanned and sliced as described in the Materials and Methods section. The \(^3\)H and \(^14\)C d.p.m. in each slice was calculated as a percentage of the total d.p.m. recovered from the gel. The distribution of \(^3\)H-labelled nRNA for control rats is shown by ---- and that of \(^14\)C labelled nRNA of rats recovering from convulsions by ---. The percentage change in the amounts of \(^14\)C-labelled nRNA relative to the \(^3\)H (O) was calculated by

\[
\frac{\text{\(^3\)H/\(^14\)C of each slice}}{\text{\(^3\)H/\(^14\)C of the RNA sample loaded on the gel}} \times 100
\]

The position of 28S and 18S rRNA is indicated. (a) After 15 min labelling *in vivo*, RNA (60\,\mu g/60\,\mu l) containing 113 400 d.p.m. of \(^3\)H and 6120 d.p.m. of \(^14\)C was loaded on the gel with recoveries of 103 and 108\% respectively. (b) After 30 min labelling *in vivo*, RNA (100\,\mu g/60\,\mu l) containing 336 550 d.p.m. of \(^3\)H and 24 770 d.p.m. of \(^14\)C was loaded on the gel with recoveries of 104\% for both labels.
they were scanned at 265 nm and then cut into slices of 1 or 2 nm thickness. A very heterogeneous pattern of rapidly labelled RNA was seen (Figs. 4–6). This heterogeneity has been observed many times in brain and other tissues (Saborio & Aleman, 1970; Scherrer et al., 1966). With 1 mm slices, numerous small peaks were detected. Some of these peaks, but certainly not all, could be accounted for by the error in the gel-slicing machine (±2.2%, see the Materials and Methods section). The labelling pattern of duplicate gels showed some minor variation in the heights of the peaks, although the overall shape was the same.

In general no sharp differences in the amount of any one specific size of HnRNA molecules were found for convulsed rats. The overall distribution of $^3$H- and $^{14}$C-labelled HnRNA was similar for each of the six different times. What was detectable was the rate of change in the relative amounts of labelled HnRNA of convulsed and control rats, in each slice measured by the $^{3}$H/$^{14}$C ratio. The $^{3}$H/$^{14}$C ratio for each slice was expressed as a percentage of the $^{3}$H/$^{14}$C ratio of the purified RNA loaded on the gel. Thus a value higher than 100% represents an enrichment of the $^{14}$C d.p.m. in that fraction relative to the total RNA.

Fig. 5. Labelling pattern of nuclear RNA from normal and convulsed rats

(a) Percentage distribution at 1 h after injections and convulsions. nRNA (50 $\mu$g/30 $\mu$l) contained 373 550 d.p.m. of $^3$H and 46 520 d.p.m. of $^{14}$C with recoveries of 112 and 110% respectively. (b) Percentage distribution 14 h after injections and convulsions. nRNA (50 $\mu$g/50 $\mu$l) contained 177 820 d.p.m. of $^3$H and 41 930 d.p.m. of $^{14}$C with recoveries from the gel of 96 and 102% respectively. All other details and symbols are described in Fig. 4.
and a value of less than 100% represents a deficit in $^{14}$C d.p.m. in that fraction. This was a direct measure of the comparative rate of processing of the HnRNA in rats recovering from seizures.

At 15 min, there was a 5–18% enrichment in the giant-sized $^{14}$C-labelled RNA molecules up to fraction 16 in the convulsed rats (Fig. 4a). For fractions beyond this, the rate of processing appeared to be normal, except for the smaller-sized RNA (fractions 31–38), where again there was a slight enrichment in $^{14}$C-labelled RNA. However, at 30 min, there was a very marked change (Fig. 4b). Although synthesis was still continuing, the rate of processing had decreased considerably in the convulsed rats. A 10% excess of giant-sized $^{14}$C-labelled HnRNA was found in all slices up to 18, followed by a very rapid decline to 70% of the expected value (fraction 30) and then a decreased low amount of smaller $^{14}$C-labelled RNA species. Some measure of the degree of inhibition of the processing could be gauged from the rate of the percentage changes of $^{3}$H/$^{14}$C ratio along the length of the gel. The line of best fit was calculated for fractions 17–30 and gave a slope of 2.60.

At 1 h, the processing was still strongly inhibited, but to a lesser extent than at 30 min (Fig. 5a). The slope per unit gel length of the percentage change for fractions 11–63 was 1.02. If the degree of inhibition...
was exactly the same as at 30 min, the gradient would have increased. There was a 20% enrichment in the giant-sized \(^{14}\)C-labelled HnRNA owing to a build-up of unprocessed molecules, although the rate of synthesis was still only 80% of the control (Fig. 2).

At 1.5 h, the rate of processing had increased, although still showing inhibition, with a gradient of 0.66, fractions 16–52 (Fig. 5b). At this time the overall rate of synthesis was 124% of the control. The same trend is continued at 2 h, the slope being 0.27 (Fig. 6a). The processing of HnRNA for convulsed rats had still not returned to normal at 6 h (Fig. 6b). The gradient of the line of best fit for fractions 13–52 was 0.35, slightly higher than at 2 h. The giant-sized \(^{14}\)C-labelled HnRNA at the top of the gel (fractions 4–15) was still present in increased amounts at 6 h. This effect could be due to a persistent inhibition of the processing rate or an inability to cope with the increased rate of incorporation at 6 h, or both.

Fig. 7 shows the result of the incubation of nRNA, labelled for 6 h, with RNAase (see the Materials and Methods section). The \(^3\)H/\(^{14}\)C ratio was constant from fraction 24 onwards. Thus all of the \(^3\)H- and \(^{14}\)C-labelled HnRNA and pre-rRNA was convertible into oligoribonucleotides. Approx. 45% of the radioactivity diffused out of the gel during fixation for 1 h.

**Labelling pattern of brain cytoplasmic RNA from control rats and those recovering from convulsions**

The purified RNA from the postnuclear supernatant of brain cerebral cortex was fractionated on 1.5% polyacrylamide–0.5% agarose gels in a fashion similar to nRNA. Amounts of up to 100–120 \(\mu\)g of cytoplasmic RNA and consequently large amounts of radioactivity could be loaded on 1.5% gels without any distortion of the 28S and 18S RNA peaks. At 1 h, \(^3\)H-labelled RNA from control rats was detected in fraction 12, well above the position of 28S RNA, with 130 d.p.m. above background (Fig. 9b). At 6 h, significant radioactivity (120 d.p.m.) was found in the same region of the gel (fraction 32, Fig. 10b). These corresponded to maximal molecular weights of \(16 \times 10^6\) and \(9 \times 10^6\). It is possible these could be ascribed to mRNA. Kumar & Lindberg (1972) found that in KB cells, 60–70% of mRNA sedimented with values above 28S on sucrose gradients, corresponding to molecular weights of up to \(5 \times 10^6\)–\(10 \times 10^6\). Ribonucleoprotein complexes cannot account for the labelling above 28S, because of the prolonged proteinase treatment.

An obvious feature of the labelling patterns of cytoplasmic RNA for both convulsed and normal rats was the high specific radioactivity and rapidity of labelling of tRNA (Figs. 8–10). It accounted for 50% of the total radioactivity of cytoplasmic RNA from 15 min to 1 h. Similar results were obtained by Saborio & Aleman (1970) for cytoplasmic fractions from rat cerebrum for labelling times of 10 min to 3 h. The rapid labelling of 4S RNA may be due to enzymically catalysed turnover of the CCA terminal triplet of the 3' end of 4S RNA. It also requires ATP. The label in cytidylic acid residues would be derived from [\(^5\)H]orotic acid, which serves as a precursor for uridylic acid. In addition some contribution from 5S RNA labelling cannot be ruled out. Leibowitz et al. (1973) have found that Ss rRNA in HeLa cells leaked out of the nuclei during isolation with labelling times up to 30 min, after which it became associated with nucleoprotein in the nucleus. The contribution of 4S labelling at 1.5 h and 2 h was 38% of the total \(^3\)H-labelled RNA, and 36–39% for \(^{14}\)C-labelled RNA. At 6 h, this had decreased to 30% for both convulsed and control rats.

The percentage distribution of labelling of cytoplasmic RNA showed a greater variability between convulsed and control rats than that of nRNA (Figs. 8–10). Some labelling in the region of 18S rRNA was detected at 15 min. It is not known whether this was mRNA or rRNA. The ratio of \(^3\)H/\(^{14}\)C was not calculated, owing to large errors resulting from low \(^{14}\)C counts. At 30 min, significant amounts of both 28S and 18S rRNA were detectable for treated and control rats (Fig. 8b). In general the amounts of newly synthesized rRNA in convulsed rats appeared to be decreased. At all time-points, the \(^3\)H/\(^{14}\)C ratio in the region of rRNA was less than 100%, and at 1 h as low as 65%, of that in the control rats.

In contrast, elevated \(^3\)H/\(^{14}\)C ratios were found for RNA larger than 28S from 30 min to 6 h. The exception was the 2h labelling pattern, where low \(^{14}\)C
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Double-labelled cytoplasmic RNA from the cerebral cortex was electrophoresed on 1.5% polyacrylamide–0.5% agarose gels for 2h and treated in the same way as for nuclear RNA. The radioactivity data were expressed in the same way as in Fig. 4. The distribution of $^3$H-labelled RNA for control rats is shown by --- and for $^{14}$C-labelled RNA of convulsed rats by -------. The percentage change in the $^3$H/$^{14}$C ratio is shown by o. (a) After 15 min labelling in vivo, RNA (133 $\mu$g/80 $\mu$l), containing 18 940 d.p.m. of $^3$H and 580 d.p.m. of $^{14}$C was applied to the gel, with recoveries of 75 and 50%, respectively. (b) After 30 min labelling in vivo, RNA (133 $\mu$g/80 $\mu$l) containing 35 280 d.p.m. of $^3$H and 1840 d.p.m. of $^{14}$C was applied to the gel with recoveries of 79 and 56%.

counts made calculations liable to error. The elevated ratios indicated that some mRNA species in the convulsed rat were present in the cytoplasm in relatively greater proportions than in the control. Because of the relatively low d.p.m., especially of $^{14}$C in some of the fractions, this conclusion must be treated with caution, since it could be argued that the higher ratio arises from a radioactivity-counting error. However, it must be pointed out that in the fast-moving fractions of mRNA containing low d.p.m., the ratios of $^3$H/$^{14}$C fluctuated in a random way, and were not consistently in one direction. It is obviously very important to provide more evidence that increased amounts of heavy mRNA were being produced in the convulsed rat by closer examination of the cytoplasmic RNA larger than 28S. Most mRNA species have been found in the region between 9S (histone and immunoglobulin mRNA) and 26S (myosin mRNA). In this region of the gel, many changes in the $^3$H/$^{14}$C ratio could be seen at each different time-point. Again this may reflect different amounts of mRNA being synthesized by rats in the post-convulsive phase, although this was partly obscured by the labelling of rRNA.

One other noticeable feature was an increase in $^{14}$C-labelled material in fractions smaller than 4S RNA for 15 min, 30 min, 1h and possibly 1½ h. This could be due to increased degradation of RNA in the
cytoplasm of convulsed rats. In contrast with mRNA, where recovery of $^3$H and $^{14}$C from the polyacrylamide gel was never lower than 95%, the recovery for cytoplasmic RNA was very low at 15 min, 75% for $^3$H and 50% for $^{14}$C, but increased with time to 91% for $^3$H and 86% for $^{14}$C at 6 h. The recovery of $^{14}$C d.p.m. was always less than $^3$H for cytoplasmic RNA run on gels. The low recovery of radioactivity from the gel is due to the presence in the preparation of cytoplasmic RNA of impurities which either fail to enter the gel because they are uncharged, or which move ahead of the 4S RNA and diffuse out of the gel during fixation. These impurities, which are labelled because of the entry of $^3$H into water and $^{14}$C into the general metabolic pools, may be removed by washing the pellets of RNA with chloroform-methanol–1 M-LiCl (5:10:4, by vol.). The recoveries of RNA from the gels then approach 100%.
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Fig. 10. Labelling pattern at 2 and 6 h of cytoplasmic RNA from normal and convulsed rats

(a) Percentage distribution at 2 h after injections and convulsions. RNA (47 μg/50 μl) containing 33530 d.p.m. of $^3$H and 2630 d.p.m. of $^{14}$C was applied to the gel, with recoveries of 93 and 90%. (b) Percentage distribution 6 h after injections and convulsions. RNA (62 μg/40 μl) containing 90650 d.p.m. of $^3$H and 15570 d.p.m. $^{14}$C was applied to the gel, with recoveries of 91 and 86% respectively. All other details and symbols are described in Fig. 8.

Discussion

Isolation of HnRNA

The method for isolation of HnRNA by using proteinase in the presence of sodium dodecyl sulphate followed by DNAase has considerable advantages over the use of phenol, which causes losses and degradation especially if a high temperature for extraction is involved. The HnRNA we obtained appears to be substantially intact, not aggregated, and is recovered in over 80% yield from nuclei. When 2M-LiCl was used to avoid DNAase incubation, large losses ensued, presumably because of the attachment of HnRNA to DNA (Price et al., 1974). Essential to the success of the method using proteinase and sodium dodecyl sulphate was the extraction of hydrophilic lipids.
These gel single the of the due & peculiar X-100 of labelled RNA to remain at the top of the gel. A similar observation was made by Peterson (1970) in the isolation of RNA from single neurons of Aplysia.

Such giant HnRNA with molecular weights up to $3 \times 10^8$ has not been reported previously and may well be peculiar to brain. In order to produce mRNA of $15 \times 10^6$ daltons, as was found in the cytoplasm, it means that only 5% of less or such HnRNA molecules is conserved.

The time required to synthesize one complete molecule of $3 \times 10^8$ daltons, i.e. $10^6$ nucleotides, would be 170min, if the rate of synthesis of HnRNA is 100 nucleotides/s (Greenberg & Penman, 1966). Our estimates of molecular weights depend on gel electrophoresis. Discrepancies between low values of the molecular weight of nRNA obtained by sucrose-density centrifugation and much higher estimates with gel electrophoresis have now been partly resolved. Bramwell (1974) has shown that the lower values were false because of conformational instability of HnRNA in low-salt gradients. Nevertheless, it is necessary to confirm the high-molecular-weight values obtained in the present work for HnRNA by other methods.

There are several reasons to justify the belief that the giant HnRNA is not an artifact of aggregation. First, there is the behaviour after treatment with dimethyl sulphoxide. Although it has been reported that this solvent can cause aggregation, this does not occur in the conditions used here (Birnboim, 1972). Secondly, cytoplasmic RNA extracted with the same method showed no such high-molecular-weight RNA (Plate 1). Thirdly, if aggregation had occurred, it would not be possible to detect changes in the $^3\text{H}/^4\text{C}$ ratio of the nRNA, as this would tend to be uniform throughout the length of the gels.

The contribution (38%) of HnRNA and pre-rRNA to the total nRNA was probably underestimated in the present work. The outer nuclear membrane with attached ribosomes was not removed with Triton X-100 because this caused clumping in brain tissue. From kinetic data, Brandhorst & McConkey (1974) have calculated that in nuclei of mouse L cells, HnRNA represents 58% and pre-rRNA 20% of the total nRNA content.

**Effect of convulsions**

It is possible that the results that we have observed were due in part to an interaction between the anaesthetic agent, halothane, and the convulsive gas flurothyl, given 4-5min after halothane. Halothane is expelled from the lungs with a half-life of 15min in rats, and probably a similar time is necessary for flurothyl. Also it is not necessarily true that the same events occur during electroconvulsive shock. Flurothyl is thought to produce a more prolonged seizure with a greater functional disturbance in the central nervous system. These points must be borne in mind in the following discussion.

A likely explanation for the fall in nRNA synthesis in the first hour is the change in size of the nucleotide pools. This will be affected by the rate of transport of orotate across the cell membrane, the conversion into UMP and the rate of phosphorylation to UDP and UTP. Prives & Quastel (1969) and Orrego (1967), investigating this problem in cortex slices, found that after electrical stimulation the uptake of uridine and its conversion into UMP was unaffected but that phosphorylation was markedly decreased. There is some evidence that total acid-soluble nucleotide pools of the cell do not bear a direct relationship to the precursor pools for nucleic acid synthesis. In Novikoff hepatoma cells, Plagemann (1972) found evidence for a small precursor pool in the nucleus. This was almost completely independent of a large expandable cytoplasmic pool which contained 95% of the ribonucleotides. This may be the situation in brain tissue, where Piccoli et al. (1969) have postulated the existence of two pools to account for the observation that UTP has the highest specific radioactivity at the earliest time after $^3\text{H}$uridine injection. Their experiments also showed that electroshock in rats increased the specific radioactivity of UTP, but at the same time that of UMP was decreased. The uridine derivatives were the only ones to show long-lasting alterations during the recovery phase after electroshock. Adenine, guanine and cytosine nucleotides underwent rapidly reversible changes. Thus the relative contribution by the specific radioactivity of the precursor ribonucleotide pools to the degree of labelling found in RNA in convulsed rats cannot be assessed at the present time. The initial decrease in labelling of RNA cannot be directly explained by the limitation of ATP. After flurothyl convulsions, brain glycogen, ATP and creatine phosphate contents fell only slightly up to 3min (Sacktor et al., 1966). Dunn (1973) showed that in mice ATP concentrations were back to normal in 2min after electroshock.

**Nuclear RNA**

In the nucleus there was a transient rapid decline in nRNA synthesis at 15min. The rate of labelling recovered quickly, so that at 1h after convulsion it was higher than in control rats. The more prolonged effect was the inhibition of processing of HnRNA evident from 30min to 6h.

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Failure to observe an effect on processing of RNA at the earliest time of 15 min was probably due to the presence of nascent RNA in the molecules of relatively low molecular weight. At subsequent points a proportionately larger fraction of label appeared in very-high-molecular-weight RNA and the inhibition of processing was then apparent. This is the first time that processing of HnRNA has been shown to be affected directly. It is difficult to propose a precise explanation, because so little is known of the control mechanisms for processing. One possibility would be that RNA polymerases initiated at the wrong positions, or inactivation of specific endonucleases and exonucleases involved in the processing may have occurred. It is known that there are large changes in cation concentrations after seizures (Ranson, 1974), which may distort normal processing. The proteins associated with HnRNA may be important in processing. It has now been shown that these proteins comprise a heterogeneous group in both size (39000-180000 mol.wt.) and charge (Pederson, 1974), and contrary to previous reports (Georgiev & Samarin, 1971) show cell specificity (Pederson, 1974).

It is thought that these proteins combine with nascent HnRNA as an early post-transcriptional event so that different HnRNA molecules are complexed with different sets of proteins. It is possible that the effect of seizures is to disrupt the interaction of these proteins with nascent RNA. Continuing protein synthesis may also be a necessary factor in the synthesis and processing of HnRNA. It has been claimed that protein synthesis was inhibited after electroshock but this was reversed after 15 min (Dunn, 1971). Poly(A) addition to HnRNA, which is a late event in processing (Perry et al., 1974), may also be affected, but it would not account for the inhibition of processing. Whatever the specific reason, the net result is a marked decrease in the number of newly synthesized RNA molecules (both mRNA and rRNA) exported from the nucleus.

An important point to bear in mind is that the continued synthesis of RNA during the isolation of nuclei, which took 5 h, was suppressed by actinomycin D, present from the moment of thawing of the tissue. The prevention of naphthalenedisulphonate was the prevention of degradation of newly synthesized RNA. It must be stressed that the effectiveness of naphthalenedisulphonate as an inhibitor of RNAase is uncertain, since penetration of the nuclei would be a prerequisite. Incubation of isolated liver nuclei, which contain greater amounts of RNAase than brain, at 38°C for 15 min resulted in 15% degradation of unlabelled RNA and 42% loss of newly synthesized nRNA into acid-soluble components (Hurlbert et al., 1973). Actinomycin D did not prevent this degradation. The results described here apply to the summation of effects in the three main classes of brain cell nuclei. Uptake of orotate has been shown to be slight-

ly dissimilar for neurons and glia after 4 h (Pohle & Matthies, 1974).

**Cytoplasmic RNA**

The maximal size of mRNA found in rat cerebral cortex was in the region of 9 x 10^6-16 x 10^6 daltons. These are rough estimates, since mobility in gels will depend on the secondary structure. Claims for mRNA of very high molecular weight have been made for sea-urchin embryos (26 S), silk gland of *Bombyx mori* (45 S), and the salivary glands of the insect *Chironomus* (75 S). To accommodate the clusters of 100 ribosomes found in isolated neurons of the lateral vestibular nucleus (Ekholm & Hyden, 1965) would require an mRNA of 3 x 10^6-5 x 10^6 daltons, assuming maximum packing of the ribosomes on the mRNA.

The time taken for the first appearance of labelled rRNA in the cytoplasm was approx. 30 min for both 18 S and 28 S species. This is in agreement with Saborio & Aleman (1970). The pattern of mRNA that was transported out of the nucleus, in the convulsed brain revealed that different mRNA species predominated at different times, as shown by the changing 3H/14C ratios. This implies that the recovery of the cells requires the synthesis of specific proteins. Interpretation is complicated by the superimposition of newly synthesized rRNA on that of the mRNA.

The results of the tRNA labelling in the cytoplasm provide an insight into the possible function of the turnover of the -CCA terminus. If the incorporation depended not on the rate of synthesis of tRNA de novo but only on the activity of tRNA nucleotidyltransferase (EC 2.7.7.25) and the size of the nucleotide pool, one would expect to see entirely different kinetics for tRNA labelling in convulsed rats. As the nucleotide pools recovered, there would have been an increase in the percentage contribution of tRNA to the total cytoplasmic labelled RNA of convulsed rats in comparison with the control, since export of RNA to the cytoplasm was still inhibited. However, this percentage was exactly the same from 15 min to 6 h for each set of control and convulsed rats. This would suggest that the turnover of -CCA is linked to the rate of appearance of newly synthesized RNA in the cytoplasm. This would also support the conjecture that this turnover serves as a simple and rapid means of switching off protein synthesis (Stent, 1964). Although this has not yet been shown for brain, decreased protein synthesis would be the expected consequence of decreased mRNA concentrations after convulsions for up to 6 h. Vesco & Giuditta (1968) found a rapid fall in the number of polyribosomes after electroshock. This was attributed to an increase in free ribosomes rather than a loss of polyribosomes produced by scission of mRNA by
RNAase (MacInnes & Luttgas, 1972). Decreased incorporation of leucine into protein was reported after a single electroshock in mice (Dunn, 1971). Both effects were short-lived, however, with protein synthesis and the monoribosome/polyribosome ratio returning to control values by 15–20 min. It could be that in contrast with electroshock, convulsions produced by flurothyl may cause much longer effects, since only 87% of the convulsant gas would be eliminated from the lungs by 45 min.

The cytoplasmic RNA prepared by our method is contaminated by glycopeptides, which may or may not be labelled. Such labelling is more likely to occur with [3H]orotate than [14C]orotate, because of H exchange with water. In convulsed rats, a large increase in 14C-labelled material was found below 4 S on the gels for preparations up to 1½ h. It is possible this represented increased breakdown of mRNA in vivo by more active RNAase molecules or that less stable mRNA was produced after seizures.

Finally, it can be noticed that there are similarities between these experiments and those performed on isolated neurons of Lymnaea stagnalis and Aplysia californica, after electrical and synaptic stimulation (Bocharova et al., 1972; Peterson, 1973). During stimulation there was a decrease in incorporation of 3H-uridine, followed by a marked increase in incorporation of up to 200%. However, labelled RNA was completely absent from the cytoplasm until 1½ h after the onset of stimulation.

It is not known whether these experiments reflect functional changes of neurons participating in learning and memory storage or rather gross disturbances of the normal metabolic processes. Certainly the convulsive effects in brain provide a tool for examining the factors controlling processing of HnRNA and release of mRNA from the nucleus to the cytoplasm.

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