NEUROCHEMICAL CORRELATES OF LEARNING AND ENVIRONMENTAL CHANGE

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1. Introduction

It is common ground amongst all approaches to the problem of learning that the learning process must induce a change of state of the brain of the learning animal, so that the brain of the animal having learned differs in some - hopefully measurable - manner from that of the naive animal. Because of the durability of long-term memories, once fixed, it seems probable that this change of state is associated with specific changes in the physical-chemical-anatomical matrix of the brain. The well-documented resistance of brain memories to physical, chemical or electrical insult to the brain in the form of damage or lesions, centrally acting drugs, lack of essential metabolites, electro-convulsive shock or interruption of electrical activity, speak for themselves. Indeed both in humans and experimental animals, it is extremely difficult to expunge memories at all, short of totally removing the cortex. Additional support for this hypothesis comes from the considerable data concerning the plasticity of this physical-chemical-anatomical matrix of the brain during exposure to conditions ranging from the developmental to the pathological, which will, in part, be reviewed below.

It is necessary to identify the substances which change, in composition or amount, during memory storage, and their cellular and physiological significance. The criteria for accepting particular substances as intermediates in a complex biological process were laid down several years ago now by Slater (Adv. Enz. 20 (1958) 147) with respect to the intermediates of electron transport. A suspected intermediate in such a sequence must - a) be present in stoichiometric quantities within the tissue; changes in the tissue capacity for conducting the reaction must be correlated with changes in the intermediate. b) Selective inhibition of the intermediate must inhibit the process and removal of the inhibitor must reactivate. (The reciprocal, that activation of the intermediate must speed up the process, does not necessarily follow unless the intermediate is rate-limiting in quantity.) c) The removal of the intermediate must specifically inactivate the process; its re-addition must selectively reactivate it. The nature of the criteria does not change, whether the process under investigation is memory or electron transport. Experiments designed to search for the intermediates along the lines of all three of these criteria have been made in the study of memory mechanisms. Amongst the classes of change, detectable by biochemical means, that might be associated with learning one could potentially include (i) changes in the cellular distribution of preformed substances, (ii) changes in the transport properties of the cellular membrane, (iii) changes in the amount or nature of transmitters at the synapse, (iv) changes in extracellular structures at the synaptic cleft, (v) changes in the activity of enzyme systems either free, or more likely, membrane-bound. Detection of such changes could involve measurement of the levels of low molecular weight substances like amino acids and amines, lipids, lipoproteins, mucopolysaccharides or proteins. Yet the body of research in this field has concentrated on detection of changes in the amounts or rates of production of two general classes of macromolecule, protein and RNA.

This parsimony may result from the prevailing *zeitgeist* of molecular biology, especially when studies in this field began seriously a decade or so ago. A par-

ticular semantic confusion, occasioned by the deliberate use on the part of geneticists of the phrase "genetic memory" and by immunologists of "immunological memory" and of DNA, RNA, and protein as "informational macromolecules" which coded these ended in the analogy being turned back on itself. Brain memory, too, was seen as a function of the unique coding properties of the nucleic acids. This idea found expression in an early hypothesis of Hydén (in The Cell, ed. Brachet and Mirsky, IV, 215–325, Academic Press, New York, 1969); (he has subsequently modified this hypothesis, though; see, e.g. Hyden in The Human Mind, ed. Roslansky, pp 29–61, North Holland, Amsterdam, 1968).

In its most simplistic form this model is expressed, as in a recent paper by Mekler: - "There are known to be three types of biological memory: (a) genetic memory, the discovery and unravelling of which has been due to molecular biology; (b) conventional memory, which is a function of the brain; and (c) immunological memory. In spite of the apparent differences between these types of memory they probably have much in common, and presumably a single mechanism is responsible for the working of all three". (Mekler, Nature 215, (1967) 481). That either RNA or protein molecules, by virtue of their unique coding potential, themselves represent individual memory traces, however, is on closer inspection scarcely to be regarded as probable. Such a role would seem to make redundant the known coding properties of the neuronal network within the central nervous system, with its pattern of synaptic interconnections making possible an intercellular, rather than a intracellular, information storage system. At the same time it would load yet another function on to the - already heavily oversubscribed - protein synthesizing apparatus of the cell. It is to be noted though, that the "transfer" class of experiments, which represent an application of Slater's third criterion, are essentially a test of just this hypothesis, although the views of its protagonists as to how the molecule/memory-trace equivalance operates have not yet been clearly presented. Controversy surrounding the meaning of these "transfer of learning by injection" experiments makes it difficult to deal with them adequately in a short space, and they will not be discussed further here (for reviews, see Ungar, Perspect. Biol. Med., in press, 1969; Rose in Applied Neurochemistry, ed. Davison & Dobbing pp 356–376, Blackwells, Oxford, 1968). Nonetheless, the power of the brain memory/genetic memory analogy has served to attention on changes in protein and RNA synthesis on consolidation of memory. There would indeed be good grounds for anticipating that, whatever the proximate biochemical mechanisms involved in memory fixation, changes in the rates of production of RNA and protein would be amongst the mechanisms involved, in common with many other cellular control systems, such as, for instance, hormone action.

2. Changes in amount of RNA and protein within the brain

Under this heading are grouped the experiments designed to test the involvement of RNA and protein in brain processes under Slater's first criterion (see above). Two approaches, long and short-term, are possible, though both have limitations.

(a) *Responses to long-term alterations in the environment.* The most detailed studies in this category that are purely biochemical in their analysis have been made by Bennett, Krech, Rosenzweig and their coworkers (Physiol. and Behav. 1, (1966) 99 and 3, (1968) 819; J. Comp. Physiol. Psychol. 55 (1962) 429). This group uses an experimental situation in which rats are reared either in conditions of environmental complexity, in which the animals live together in a colony, have a variety of "toys" and mazes with which to experiment, and are frequently handled; or in conditions of greater or lesser isolation, with individual cages, diffuse and subdued lighting and controlled sound, and little or no handling. After a period of 80 days, the brains were samples for a number of variables, including cholinesterase levels, brain weight and cortical thickness. Typically there appeared to be an increase in the cortical weight and thickness up to 10% in the environmentally enriched group compared to their impoverished controls, whilst AchE activity decreased, especially in the visual cortex. By contrast the enzyme increased, at least in total quantity, in the subcortical regions. As little as one hour's exposure daily to the enriched conditions was sufficient to produce a statistically measurable change. Whilst there were differences in other aspects of the animal's condition at the end of these ex-

posures, for instance, in both adrenal and total body weights, this group consider the data to reflect real changes in brain chemistry and anatomy consequent upon the environmental situation.

Related to these observations are the histochemical studies of Gyllensten, who has examined the visual cortex of mice reared in the dark and observed it to be less well development than that of normally reared litter-mates, in terms of reduced intranuclear distance, consequent upon a diminution in dendritic branching (Gyllensten, Malmfors and Norrlin, J. Comp. Neurol. 124 (1965) 149).

It is difficult to relate these changes more than inferentially to the behavioural effects of such treatments as light deprivation or exposure to enriched environments. To link them more closely it is necessary to examine changes occuring over a time-scale more in keeping with that known to operate during memory fixation - i.e. periods in the range of minutes to hours (McGaugh, Science, 153 (1966) 1351).

(b) *Short term changes in RNA and protein metabolism.* The best known work in this category is almost certainly that of Hydén (for reviews, see above). These studies have depended on the use of two techniques; a microdissection method for separation of small numbers of individual neurons and their surrounding glia, (a refinement makes possible the separation of nuclei from the cells), and a micro-analytical system for the absolute amounts of RNA present in individual cells and its composition in terms of the percentage of its constituent nucleotide bases.

Hydén used this technique to demonstrate that the total amount of RNA present, in both neurons and glia, seemed to be dependent upon the behavioural status of the animal. Thus "passive" stimulation of a rabbit by periods of rotation over several days resulted in an increase in the protein of Deiter's nucleus neurons of some 12%, and of RNA by 4%. The RNA content of the glia was simultaneously reduced by 31%. More recently Jarlstedt has observed similar changes in certain of the Purkinje neurons of the cerebellum (Acta Physiol. Scand. 67, supp 271, 1966).

In these "passive stimulation" experiments Hydén found that the composition of the RNA of the neurons was unaltered, despite an increase in total amount. This he contrasted with the situation during "active learning", for example, by hungry rats, forced to learn to balance up a steel wire at 45° slope to reach food. After 4 days training Deiter's nucleus neurons and their nuclei were examined for changes in RNA levels and base ratios compared to inactive or passively stimulated (rotated) controls. For the neurons, as well as an increase of 10% in total RNA, the base ratios of the nuclear RNA were altered, the ratio of adenine increasing from 21.4 to 24.1% and that of uracil decreasing from 20.5 to 18.2%. Such a change represents a shift in the composition of the RNA; it becomes more DNA-like. This change would be anticipated if the result of the behavioural situation was to increase the synthesis of messenger-RNA. Later, parallel changes were observed in the glia also, although here total and not merely nuclear RNA was measured, and this change occured presumably contemporaneously with the decline in total glial RNA.

More recently the experimental situation has been changed to one involving transfer of handedness in a spontaneously right-pawed rat forced to reach for food with its left paw. Here the cortical neurons from layers 5 and 6 were used on the right side, and as controls, neurons from the identical region of the left side were chosen. Again, in six animals increases in total RNA and adenine and a reduction in cytosine were found. (Hydén and Egyházi, Proc. Nat. Acad. Sci. 52 (1964) 1030), although the response varied with the duration of the learning experience (Hydén and Lange, Proc. Nat. Acad. Sci. 53 (1965) 946). In similar experiments, Hydén has more recently used isotopic tracer techniques to examine changes in the rate of RNA protein synthesis as well, and finds increases in the rate of incorporation of labelled orotic acid into RNA and amino acid into protein in the hippocampus (Hydén and Lange, Science 159 (1968) 1370). Very recently he has claimed that a specific new acidic protein species, identified by its position on polyacrylamide gel separation, appears during learning (Hydén, Febs. Anstr. Madrid No. 79 1969).

Hydén's results have been criticised for the statistical validity of the analysis performed, which use relatively small sample numbers (Bowman and Harding, Science 164 (1969) 199), the exact behavioural significance of the neurons chosen for analysis, particularly in the transfer of handedness experiments (Kuffler and Nicholls, Ergenbn. der physiol. 57 (1966) 1) and because the changes observed by Hydén, of increase in RNA, etc. ought presumably to be transient. Thus, when a totally new protein species is obtained

as in the experiments using the transfer of handedness paradigm and the hippocampal region (Hydén, Febs Abstr. Madrid, No. 79, 1969) ought it not subsequently to disappear once more when the new learned behaviour is established? Otherwise it should be present already on the control side which has presumably already coded the particular behaviour studied. In addition, it is doubtful whether the hypothesis that increased messenger synthesis results only from "active learning" whilst "passive stimulation" produces changes only in total (i.e. messenger plus ribosomal, plus transfer RNA) can be sustained in the light of the fact that Jarlstedt, using similar "passive stimulation" techniques of rotation, or of caloric stimulation by irrigating a rabbit's ear with warm water, finds base ratio changes in neurons of the cerebellum (lobulus centralis and nodulus) similar to those observed during "active learning" by Hydén.

Nonetheless, the broader conclusion of Hydén's experiments, that various behavioural manipulations in an experimental animal can result in changes in the rates of synthesis and the total amounts of RNA and protein present in specific regions of the central nervous system, would seem substantiated. Evidence from several other laboratories points in this direction. The autoradiographic data of Watson (J. Physiol. 180 (1965) 754) for instance, indicate that stimulation of the supra-optic neurons by the substitution of aqueous solutions of sodium chloride for drinking water for periods of 1–60 days, results in a transient increase in the apparent rate of transfer of RNA from nucleus to cytoplasm, as measured by counting grain densities. This again, though, represents a "passive" rather than an "active" effect, and similar changes occur, scarcely suprisingly, in regeneration and repair following nerve section or crush (Lambert and Danehold, in Macromolecules & The Function of the Neuron, ed. Lodin and Rose, pp 334–43, Excerpta Medica, Amsterdam, 1968; Sjöstrand, Acta Physiol Scand. 67 (1966) supp. 270).

Specific learning effects have been claimed by Zemp, Glassman and their coworkers using shock avoidance situations and yoke controls. In Zemp's experiments, mice injected with labelled uracil as an RNA precursor were trained to jump to a shelf from an electrified grid in response to a buzzer and flashing light. The yoke controls were presented randomly with buzzer, light and shock. Compared to the yoke controls, 6.5–119% more label was found in the nuclear RNA and 7–180% more in ribosomal RNA in the learning animals. These differences were confined to the brain and were most marked in the diecephalon, no effects being found in liver and kidney RNA. (Zemp, Wilson, Schlesinger, Boggan and Glassman, Proc. Nat. Acad. Sci. 55 (1966) 1423; Adair, Wilson and Glassman, Proc. Nat. Acad. Sci. 61 (1968) 606, 917). Remarkably, there was claimed to be no difference between the yoke control animals and unstimulated controls (although this conclusion is based on experiments with only 4 pairs of animals). The use of double-labelling techniques for biochemical part of these analyses is a neat way of reducing experimental error, and the size of the effect observed by Zemp et al., is most striking.

More recently, confirmation of it has come in rather similar experiments by Machlus and Gaito (Nature, 222 (1969) 573), who have used the sensitive measures of DNA-RNA hybridization to detect the production of new DNA-like RNA in a shock avoidance situation in rats. Once again, the controls were yoked. This study showed that in the trained, but not the control animals, there was an increased production of new DNA-like RNA. This is generally taken as an indication that the new RNA is messenger. Machlus and Gaito claim that these observations imply the production of an RNA species "unique" to learning, but this conclusion is not, in my view, borne out by their data, which merely show that the RNA is a messenger, and that it is not present in the controls. Such messenger could presumably be produced under a variety of different conditions; only sequence determination, or an analysis of the protein for which the messenger codes, could show how far it would be proper to regard the RNA as "unique" in the Machlus and Gaito sense. In addition, the use of yoke controls, powerful though it is, does not entirely rule out the intervention of alternative effects to learning in the experimental animal. Indeed, one of the more surprising features of this group of experiments in view of the observations already referred to, concerning the ease with which non-specific effects apparently alter RNA production in the central nervous system, is that the authors claimed to find no difference between yoke control and quiet (unstimulated) control animals. The data of Hydén, of Jarlstedt, and of our own experiments (see below) lead one to expect that some differences would occur between these two situations.

Our own experiments relate to the effects of light

stimulation. In an early series, rats were raised from birth until 7 weeks in light-tight boxes. At this age, half of each litter were withdrawn from the box and exposed to continuous laboratory illumination, and the rate of incorporation ^{3}H-lysine into protein into various brain regions followed. The rate of incorporation of amino acid into visual cortex proteins was increased by 15–20% within 3 hours after the emergence of the animals into the light. This effects was specific to the visual cortex; other brain regions showed no effect or (motor cortex) a significant decline in incorporation. In the liver no effect was observed. The effect was a transient response to a new situation. Increasing the light exposure to 6 hrs abolished the stimulation, whilst periods of 9–99 hours resulted in a depression of incorporation of 20–35% compared with the controls. By 99 hours the incorporation was back to normal once more. By contrast, the effect in the motor cortex was consistently one of depression, also coming back to normal after 99 hours, whilst in the liver no effect occurred at any time (Rose, Nature, 215 (1967) 253). This biphasic effect may serve to unify the rather conflicting reports in the literature on the effect of varying types of stimulation and stress on protein synthesis. Thus in contrast to the stimulation we described initially, Jakoubek and Gutmann (In Macromolecules and The Function of the Neuron, ed. Lodin & Rose, pp 285–296, Excerpta Medica, Amsterdam, 1968) observed a decrease in protein synthesis in spinal motorneurons of rats following 90 min of enforced exercise (swimming). Altman and Das (Physiol. Behav. 1 (1966) 105) and Altman, Das and Chang (Physiol. Behaviour 1 (1966) 111) failed to find any change in incorporation of leucine into brain proteins, using autoradiographic techniques, following a series of stimulations including forced exercise, visual training and enriched environment. Metzger, Cuenod, Grynbaum and Waelsch, (J. Neurochem, 14 (1967) 183) found no change in the rate of incorporation of precursors into protein during unilateral stimulation with flashing light in the split brain monkey, whilst in unoperated monkeys and rabbits Talwar et al. (Talwar, Chopra, Goel & D'Monte, J. Neurochem. 13 (1966) 109; Singh & Talwar, J. Neurochem. 16 (1969) 951) have found substantial increase in lysine incorporation compared to controls following exposure to a flashing light. It is thus clear that the effects measured are both labile and reversible, dependent upon the nature and duration of the stimuli chosen. Only in few of these experiments has a full time course of the effect been analysed. Possibly the divergencies depend upon reversals or saturations of the type we have shown.

In order to discriminate further between situations involving learning and those involving relatively non-specific sensory stimulation, we turned from the rat to an alternative experimental animal, the young chick. The experimental situation was an examination of changes in protein and RNA synthesis in the brain of the 1-day old chick during the process known as "imprinting" - that process by which a bird's preference for a particular object becomes restricted to that object - an effect which occurs in many precocial birds (for review see Bateson, Biol. Rev. 41 (1966) 177). This situation is admittedly complicated in that any effects of experience on the nervous system are superimposed upon maturational changes that proceed in the absence of such experience. Nevertheless, its potential advantages are that the previous experience of the animal is very limited, the initial stages of the learning process are rapid, and in the absence of appropriate stimulation the development of a preference can be delayed. In addition, a powerful biochemical advantage of an experimental system involving the use very young animals is the almost complete absence of a blood-brain barrier in them.

In these experiments, incorporation of tritiated lysine or uracil over 38–150 min in birds during imprinting on a prominent object (a flashing light), was compared to that in birds exposed to diffuse light and in the dark. The effectiveness of the imprinting was tested after exposure by placing the birds in an alley in which they could choose to approach the flashing light. Incorporation was studied in three brain regions and the liver. Because the imprinting effect is age-dependent, groups of birds of different post-hatch age were examined. In the young birds a significantly increased incorporation of lysine into protein occured after 150 min in one brain region, the forebrain roof, compared to either light or dark controls ($p < 0.05$). In other brain regions, and the liver, there was no significant difference, nor was there in the older birds. When incorporation of uracil was examined, 76 min after exposure to the flashing light, the specific activity of the RNA in the forebrain roof was significantly raised above the control. However, more prolonged exposure (150 min) resulted in significantly in-

creased incorporation in all brain regions in the light control as well as the experimental animals when compared to the dark control ($p < 0.001$) with the light control birds midway in level between experimental and dark control. These effects suggest that a complex situation pertains; it is possible that part of the changed levels observed were specific to the imprinting situation, part to the non-specific light stimulation; the specific changes seemed to be time-dependent (Bateson, Horn & Rose, Nature, 223 (1969) 534; Rose, Horn, Horn & Bateson, Nature, in Press). The further details of this time-course may prove of interest to examine.

3. Effects of inhibitors of protein and RNA synthesis

The effects of metabolic inhibitors on learning may be studied under the second of the Slater criteria referred to above, although early work in this field has utilised such inhibitors of RNA synthesis as the purine analoque 8-azaguanine, most of the recent studies have involved two inhibitors of protein synthesis, puromycin and acetoxycycloheximide (AXM). The mode of action of those two agents is different, puromycin functioning as a chain terminator for peptides on the ribosome, where it becomes incorporated into nascent proteins disrupting their formation and resulting in the release of puromycin-peptides, and AXM preventing the expression of new messenger RNA. The use of these agents has been reviewed (Agranoff, in The Neurosciences, ed., Quarton, Melnechuk and Schmitt, pp 756–762, Rockefeller Press, New York, 1967; Booth, Psychol. Bull. 68 (1967) 149). Agranoff's studies have utilized puromycin injected intracranially in goldfish trained in shuttle boxes with light paired with electric shock, an avoidance task which the fish learn reliably. Following 20 trials over 40 minutes on day 1, the fish were retested for retention on day 4. Puromycin injected intracranially immediately after the first series of trials produced a significant loss of retention on retesting. This effect was time-dependent; if injection was delayed to 60 min after the trial series there was no deficit. It was also dose-dependent - 170 μg of puromycin was necessary for total loss of retention. Puromycin injected immediately pretrial had no effect on the learning but resulted in memory deficit on day 4. These results seem convincing, and were confirmed by later experiments in which AXM was found to produce similar deficits at concentrations one hundredth of those required for puromycin. (Brink, Davis and Agranoff, J. Neurochem. 13 (1966) 889).

Yet the result are not easily extrapolated to the mammal. Thus in mice 80–90% of cerebral protein biosynthesis can be inhibited for 8–10 hours by bilateral temporal injection of puromycin. Using this technique, Flexner and his colleagues were able to obtain loss of recent memory in mice injected within 24 hrs of training on a Y-maze. On the other hand, chloramphenicol, which produced inhibition lasting less than 4 hrs, was without effect on retention (Flexner, Flexner, Roberts and De La Haba, Proc. Nat. Acad. Sci. 52 (1964) 1165). Nor did AXM injected either before, during or after training appear to affect long-term retention. Instead there was a complex situation in which there was an intermediate period lasting several hours during which the memory was lost, and an eventual return. What is more, it seems as if AXM antagonised the puromycin effect. Injected in conjunction with puromycin, it prevented the puromycin-induced memory loss (Flexner, Flexner, Proc. Nat. Acad. Sci. 55 (1965) 369); Flexner, Flexner and Roberts, Proc. Nat. Acad. Sci. 56 (1966) 730). Such results have been explained by Flexner on the basis of the different modes of action of the two inhibitors. AXM merely delays the appearance of the messenger, whilst puromycin destroys the message by producing nonsense peptides on the ribosome. AXM thus counteracts the puromycin by delaying the appearance of the new messenger until puromycin-induced nonsense-peptide formation has ceased. These experiments imply a three-stage process in memory consolidation, an early phase which is independent of protein synthesis, an intermediate phase in which the expression of the memory demands messenger RNA production and the final stabilisation of the memory trace only several days subsequently. All of these results have been made more confusing by the more recent observation by Flexner and Flexner (Science, 159 (1968) 320), that intracerebral injections of normal saline are adequate to remove the blockage of memory resulting from puromycin injected some time after training, though not that resulting from puromycin injected before or during training. In the former case, the memory could apparently be restored

as long as 60 days after the puromycin injection. These surprising results have suggested to Flexner that there is a double effect caused by puromycin, an immediate inhibition of the type proposed above, and one caused by the long-term persistence in the brain of puromycin-peptides which cause secondary memory loss (Flexner and Flexner, Proc. Nat. Acad. Sci. 60 (1968) 923).

Such complexities demonstrate one of the major difficulties in the way of unequivocal assessment of data derived from the use of inhibitors of biochemical processes, that is, the problem of distinguishing between primary and secondary effects of the agent. Thus both behavioural and biochemical specificity may be suspect. Although the major known effect of such agents as puromycin and AXM is on protein synthesis, others may also occur, which are responsible for the measured behavioural deficits, yet have not been observed, because they are not relevant in the non-behavioural situations in which the agents are generally used. Thus it may be of interest that puromycin, for instance, has been shown to affect the morphology of neuronal mitochondria (Gambetti, Gonatas and Flexner, Science 161 (1968) 900) whilst puromycin, but not AXM, will cause hippocampal seizures (Cohen, Ervin and Barondes, Science 154 (1966) 1557). In addition, it must be pointed out that puromycin is very toxic. Doses twice as high as those used by Agranoff will cause death in goldfish, whilst in mice, doses only slightly above those applied in the behavioural experiments will cause disorientation, convulsions and death, (Flexner, Flexner, Stellar, De La Haba and Roberts, J. Neurochem. 9 (1962) 595). Under the circumstances, it is perhaps even more surprising that the only observable deficit in an experimental animal in response to an agent which blocks 90% of protein synthesis in the brain for periods of several hours should be an inability to fix recent memory.

In answer to such criticisms, Agranoff has pointed out (Agranoff in The Neurosciences, ed. Quarton, Melnechuk and Schmitt, pp 756–762, Rockefeller Press, New York, 1967) that in a sense the complexity of the behavioural relationship between puromycin and AXM is encouraging, as the only known interaction of the two is in their effects on protein synthesis. Hence the effect of AXM on puromycin in the mouse is supportive evidence that the puromycin effect is related to the inhibition of protein synthesis. But it seems more probable that a valuable role for the inhibitor studies will be found by including them amongst those agents which, by affecting memory consolidation, can be used to investigate the time-course of fixation, rather than that they will reveal much more about the biochemical mechanisms concerned. In this sense, the agents take their place alongside such agents as electroshock or metrazol (McGaugh, Science 153 (1966) 1351) or pemoline (Orzack, Taylor and Kornetsky, Psychopharm. 13 (1968) 413) or nicotine (Oliveiro, Life Sci. 7 (1968) 1163) and it is in this manner that they have found most recent use (Davis and Agranoff, Proc. Nat. Acad. Sci. 55 (1966) 555; Barondes and Cohen, Proc. Nat. Acad. Sci. 61 (1968) 923).

4. Possible models

Thus it would seem proper at this stage to make a strictly minimalist statement of the biochemical position: that a variety of behavioural events resulting from environmental stimuli produce a situation in which, in specific brain regions, possibly localised to within a relatively limited number of cells, a biochemical "trigger" is pulled which results in the synthesis, first, of new messenger RNA, and subsequently, of new protein. In this sense, RNA and protein metabolism within the brain are state-dependent. Whether this protein is of a fundamentally new species or is present previously in the cell but in lower quantities, is uncertain. The role of the new protein is not known, and it is not necessary to assume that in some form it represents a chemical engram for the memory; it may in its turn be an enzyme for the formation of other biochemical species.

Whilst excessive theorizing is premature, it would seem probable that the observed biochemical changes thus represent one aspect of an overall metabolic and morphological change at the cellular level which occurs in response to the environmental stimulus. What sort of change might be involved is a matter for speculation, as the localisation (e.g. neuronal versus glial) time course and specificity of the changed rates of synthesis are not known in enough detail.

Most current models, though, specify that the biochemical change occurs at the synaptic level, by modification, for instance, of a previously existing synapse. This synapse may be excitatory or inhibitory,

and the effect of the modification could equally well be to activate as to inhibit it. This modification (which might involve pre-synaptic or post-synaptic changes, or alterations in the synaptic cleft) could involve the following sequence of events:

(a) environmental trigger.
(b) unique constellation of inputs to particular cell(s).
(c) stimulation of m-RNA synthesis.
(d) new protein synthesis.
(e) transport of protein down axon to synapse (axoplasmic flow).
(f) at the synapse, modification of existing structures by incorporation of protein into membrane; its enzyme role in synthesis of new metabolites, etc.
(g) development of homeostatic mechanism to ensure that despite subsequent turnover of the protein the metabolic and morphological change of state remains unimpaired.

The time-course of such a sequence of events is of obvious interest here, and it is of relevance that the two key steps, those of protein synthesis and of axoplasmic flow, can be shown to occur with time constants of the order of minutes to hours, i.e. constants appropriate to those known to operate in memory fixation. Note, however, that such a sequence does not say anything about whether a single or many cells are modified, whether they are spatially contiguous or widely separated within the brain, or the detailed effect of the resulting synaptic modification. Nor are we here called upon to postulate mechanisms whereby such a changed structure or biochemistry of the synapse can result in recall; the hypothesis relates only to the events of information storage, not of retrieval.

Several points in this sequence are now amenable to experimental attack. Key areas for experiment would currently seem to include points (c), (d) and (f) above. At the biochemical level, the species of new protein being produced need identification, and any potential synaptic role for them needs examination. At the morphological level one may ask whether any identifiable synaptic changes can, in fact, be observed in learning animals.

Partly the answer to these questions will depend on whether one is considering learning in the developing or in the adult brain. Obviously during development new synapse formation is occuring continuously; some of this may be in response to genetically specified wiring instructions; that some is in response to the changed environmental situation would be implied by the experiments of Bennet et al. that were discussed above. That synaptic modifications is, however, possible even in the adult animal is clear from the observations of Cragg (Nature, 215 (1967) 251; Brain Res. in Press, 1969) of Globus and Scheibel (Exp. Neurol. 19 (1967) 331) and of Valverde (Exp. Brain Res. 3 (1967) 337) and Valverde and Ruiz Marcos, (Febs Abstr., Madrid, No. 73, 1969) for instance. Environmental deprivation and stimulation result in changes in the numbers and size of dendritic branches and in the numbers and diameters of synapses in the visual cortex of rats during first exposure to light over a time-course compatible with the changes in protein synthesis that we have observed. Such observations suggest that the way may be clear for a combined biochemical-morphological-neurophysiological approach to brain mechanisms in learning. No one brain discipline, though, can achive this in isolation.

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