The Storage of Acetylcholine in Presynaptic Nerve Terminals
The Scientific Basis of Medicine

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<Opening titles>

<Gilliland to camera>

Dr Victor Whittaker is the reader in biochemistry at Cambridge University. His systematic work in biochemistry is well known. In particular, his work on synaptosomes, subcellular chemical transmitters, has attracted international attention. This meticulous, logical, scientific work forms the subject of today's discourse. Dr Whittaker.

<Whittaker, seated, to camera>

There are estimated to be about 10^{14} functional contacts or synapses between nerve cells in the human central nervous system and an additional undetermined number in the peripheral nervous system in autonomic ganglia and between nerves and muscles. In the vast majority of synapses, transmission is believed to be brought

about chemically by the release of a chemical transmitter substance from the presynaptic nerve terminal in response to the arrival of an action potential in the terminal region.

This arrangement has certain important biological advantages. It ensures uni-directionality of transmission, probably essential for the ordinate processing of information, because only in the presynaptic terminal do we find the stores of transmitter necessary for transmission to take place. Then again it's difficult to imagine how synaptic inhibition, a process just as important as excitation, can be brought about by purely electrical means. It's much simpler to imagine it being brought about by chemical means by the opening of gates for ions in the postsynaptic cell or by metabolic effects.

Yet another reason for chemical transmission may be to prevent unwanted crosstalk between intertwined but functionally distinct portions of the central nervous system. There is some evidence that different systems, for example the limbic system concerned with the effective state of the individual or the ascending reticular activation system concerned with awareness and attention, may use different transmitters, so that we can think of different neurons or different pathways as being chemically coded and evolving postsynaptic receptors that are specifically sensitive to one kind of transmitter or another.

The evolutionary advantages of chemical transmission, however, exact a price. The synapse is vulnerable to a large number of substances that are sufficiently close in chemical structure to the natural transmitters to gain access to the synaptic region, but insufficiently close in structure to deputise for them and behave in the same way functionally. Such substances may sit on the postsynaptic receptors and block transmission in that way or displace the transmitter from the presynaptic storage sites. Other drugs may block the reutilisation of transmitters or may inhibit their breakdown in activation when their job is done. Yet others may block the coupling between stimulation and release of transmitter.

Natural defects in the release and storage mechanism may occur as in the disease myasthenia gravis where there is a failure of chemical transmission at the neuromuscular junction. One theory of the aetiology of manic-depressive psychoses and of schizophrenia postulates that defects in monoamine transmission in the central nervous system are involved. Whether or not this is true, it is certainly true that many drugs that act on the central nervous system either as hallucinogens or tranquillisers are chemical analogues of central transmitters.

I've got three slides here which illustrate this.

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<Whittaker narrates over projected slide>

This first one shows the formula of the transmitter dopamine which is closely related to noradrenaline. Noradrenaline has a hydroxy group on the beta-carbon atom. Below is the formula for the hallucinogen mescaline. You see that a great deal of the molecule is common in both cases. I've shown the common portion of the two molecules in molar type.

My next slide shows the transmitter 5-hydroxytryptamine which you see has a relatively simple structure with this indole nucleus here, and below it is the hallucinogen LSD – again with a common portion of the molecule. And below that again is the tranquilliser reserpine, and again you have the indole nucleus here with a certain amount of additional chemical scenery tacked on to it.

In my third slide we have yet another example – this time acetylcholine and atropine, where again you have a good deal of chemical analogy between the atropine structure and acetylcholine structure, perhaps not quite so close as in the other two examples.

<Whittaker to camera and referring to his notes>

So, you see a proper understanding of the mechanisms involved in the synthesis, storage, release and postsynaptic action of transmitters is highly relevant for neurology and psychiatry. Such problems can be investigated by currently available biochemical techniques and I now want to tell you about some recent work that I've been doing on one small aspect of this problem – the way acetylcholine is stored in nerve terminals.

Acetylcholine is a transmitter at a variety of sites in the central nervous system and many central synapses, particularly those of the ascending reticular activation system, in autonomic ganglia, in parasympathetic postganglionic fibres and at the neuromuscular junction. Ideally, the neuromuscular junction would be the best site to study cholinergic transmission biochemically because there, thanks to the classical work of Katz and co-workers using delicate micro-electrode techniques, it's at this site that we know most about the physiology of transmitter release, particularly that its release occurs in bursts or quanta of about 10,000 molecules of acetylcholine at a time.

Unfortunately, the proportion of nervous tissue and the concentration of acetylcholine in muscle is extremely small. For this reason my co-workers and I have turned to the electric organ of the torpedo.

<Whittaker narrates over projected slide >

This is a flatfish related to the ray and is an *Elasmobranchii*. The tissue in the electric organ is biologically derived from muscle with loss of the contractile element and consists, in effect, of a mass of hypertrophied endplates with a rich cholinergic nerve supply. Its acetylcholine content is several hundred times that of muscle.

<Whittaker to camera>

Our particular objective has been to isolate the synaptic vesicles present in the presynaptic nerve terminals and to find out something about their composition: in particular, how they manage to store the very high concentrations of acetylcholine

that they contain, amounting to something like 4% of the wet weight of the vesicle. We've worked with three species: *Narcine brasiliensis*, *Torpedo marmorata* and the large Atlantic *Torpedo nobiliana* which can sometimes be as much as a yard across.

<Whittaker refers to projected slide>

This picture shows *Narcine* and one can make out the two lobes of electric tissue, each side of the head. And in my next picture, I show diagrammatically the location of the organs in the fish and some details of their innervation and structure.

<Whittaker narrates over projected slide>
This is the diagram of the fish with the electric organs on each side of the head and here is a diagram of the nervous system showing the brain stem with two nuclei in which there are the cells of origin of the cholinergic fibres which run to the electric organ. And here are the four large nerve trunks containing these cholinergic fibres which run out on each side through the gill arches to the electric tissue.

The electric organ itself is made up of stacks of electroplaques which you see in this diagram. They are like stacks of pennies and they are packed together in a honeycomb structure. If one makes a vertical section through this pile, one sees each electroplaque in section as I've shown here and one finds that the lower surface of each electroplaque is profusely innervated where there are large numbers of nerve terminals applied to the under face. The top face is free from nerve terminals but is very much invaginated.

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<Whittaker to camera>

When acetylcholine is released by the synchronous discharge of the nerves, potentials analogous to the end plate potential of muscle are generated and these sum together in series to form quite a healthy charge – about 50 volts in small fish and up to 400 volts in a large *Torpedo nobiliana.* The nerve endings are just like

those anywhere else in the central nervous system as these electron micrographs show.

<Whittaker refers to a series of projected slides>

In the middle you see an actual vertical section through three electroplaques, these three here, and as you see, the innervated face is below each case. The presynaptic nerve terminals have a cytoplasm with the usual synaptic vesicles, and these nerve terminals are shown in greater detail in this electro-micrograph. You see the cytoplasm here of the presynaptic nerve terminal with the synaptic vesicles in it, and this is closely applied to this electroplaque cell here.

The vesicles are a little larger than those in muscle – they are about 840 angstroms in diameter, but as we can see in this next picture, the arrangement is quite similar to that of the familiar neuromuscular junction. Here we have the nerve terminal with synaptic vesicles in the cytoplasm and here you have the postsynaptic membrane thrown into folds, and in this case, of course, the muscle fibres below which are not present in the electric organ.

Well, breaking down the electric tissue in order to isolate the vesicles is quite a problem. The tissue is very gelatinous and it's full of collagen fibres. We found that the best approach is not to try to homogenise it using the usual Potter-Elvehjem homogeniser but instead to freeze it solid in liquid Freon-12, then powder it by crushing it in an ordinary ceramic mortar. This technique produces fragments of electroplaque cells. In electron micrographs of these shown in this picture, we can recognise the innervated and the non-innervated faces and the more or less intact presynaptic nerve terminals.

Here you have a portion of a fragment of the electroplaque with these invaginations seen in section here, which come in from the non-innervated face and here we see the nerve terminals. Now at first sight, they look reasonably intact. One can see the synaptic vesicles in situ, but a closer look at these reveals in places that a portion of the external nerve membrane which is away from the synaptic cleft has been badly

broken up leaving an exposed layer of frozen terminal cytoplasm with synaptic vesicles still embedded in it. You can see along this membrane many places where the membrane is broken or completely absent and it's clear then that this cytoplasm here with the vesicles in it is simply a layer of frozen cytoplasm which is exposed to the outside world so to speak.

I think the next picture shows this in greater detail. This is an enlargement of the portion of the previous slide and you can see here the nerve terminal with the synaptic vesicles in it, but this part of the vesicle here away from the synaptic cleft is badly broken up, exposing the frozen cytoplasm.

<Whittaker to camera>

Well, if we now stir these fragments with ice-cold sucrose solution that's been made isosmotic with the fish's plasma, the vesicles are washed off together with the soluble cytoplasmic constituents of the terminals. Centrifuging at 10,000 g for 30 minutes removes the extracted tissue fragments and leaves a supernatant string of soluble protein vesicles and a few larger membrane fragments.

We can now layer this supernatant onto a sucrose density gradient and centrifuge the vesicles away from both the larger membrane fragments and the soluble protein. To enable reasonable amounts of vesicles to be prepared, that is milligram quantities to be prepared, we use a zonal rotor. This is a type of rotor in which the separations are carried out in compartments inside the head of the rotor instead of in swinging buckets.

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<Whittaker refers to a series of projected slides>

This diagram shows a typical separation. The first peak here is a peak consisting of soluble cytoplasmic protein. Now the second peak here is a peak of bound acetylcholine. The dotted line shows the sucrose concentration in the density

gradient. Now any free acetylcholine in the supernatant is hydrolysed by the large amount of acetylcholinesterase present. The acetylcholine which we recover in this peak here has to be released from the bound state before it can be assayed. This acetylcholine can't be in the free state otherwise it would have been destroyed and it must be bound to particulate material. The way we release it is by heating fractions at an acid pH: pH 4 for ten minutes at 100 degrees. The particles with which acetylcholine is associated sediment to the gradient with a density equivalent 0.38 molar sucrose.

Electron microscopic examination of this band shows that it contains large numbers of synaptic vesicles. Now these are shown in this electron micrograph. Calculations show that there are over 40,000 molecules of acetylcholine per vesicle. This corresponds to a concentration of acetylcholine in the vesicle of about 0.4 molar – about isotonic with the torpedo plasma. This amount of acetylcholine is quite consistent with the requirements of the quantal theory of transmitter release and it's clear evidence that the vesicles are the ultimate storage site of acetylcholine within the terminals. However quite large amounts, over half of the acetylcholine in the organ, disappears during comminution and extraction. We don't know whether this is due to the disruption of vesicles or to the loss of an extra vesicular store of acetylcholine. However there is good evidence that the site of acetylcholine synthesis is in the cytosol and not in the vesicle. This is shown in my next diagram.

This is another zonal run in which the distribution of choline acetylase, the enzyme that synthesises that acetylcholine has been followed. You can see here the soluble protein peak which we saw in our previous slide and the second peak of bound acetylcholine which indicates the vesicle fraction. The distribution of the enzyme choline acetylase is shown here by these crosses and you see that the distribution is just the same as the soluble protein. There's very little of the synthetic enzyme in the region corresponding to the vesicles.

We've also shown in this diagram the distribution of the enzyme lactate dehydrogenase. This is a typical soluble cytoplasmic enzyme and you see again the distribution is similar to that of soluble protein and of choline acetylase.

So, for this reason we believe that acetylcholine must be synthesised in the cytoplasm and then later taken up by the vesicles. The vesicular character of the acetylcholine peak in these experiments is shown rather more clearly by its behaviour on gel filtration. In the top diagram, we see the effect of filtering vesicles through the gel agarose 5N, which is a gel that excludes particles of weight of 5 million or more. You can see that the acetylcholine comes through in the void volume along with most of the protein. The small amounts of protein coming out later in this peak 1 and peak 3 probably represent traces of soluble protein which have diffused from the soluble protein peak into the band containing acetylcholine.

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When this peak is rerun on the gel, you see it behaves as a single component and you see it run in the void volume.

Well, when we make corrections for the small amount of soluble protein contaminant in the vesicle preparation, we find that extremely high specific concentrations of acetylcholine are found – up to about 620 nanomoles of acetylcholine per milligram protein. This is a series of runs: this set of figures shows the actual specific concentration in the vesicle fraction as obtained from the zonal rotor in nanomoles per milligram protein. Here we have the vesicle protein as a percentage of the total protein in the preparation. This first experiment had rather a lot of contamination from the soluble protein. In these experiments we were getting rather better at the separation and the amount of contaminating soluble protein was very much less. And here we have the final corrected figure showing the true content of acetylcholine per milligram of vesicle protein.

We found that over half the protein of the vesicle preparation consists of a rather low molecular weight protein which is readily released by osmotic shock. We've named this protein, provisionally, vesiculin.

My next diagram contrasts the gel elution pattern obtained when vesicles are suspended in water with that which is obtained when they are suspended in isosmotic sucrose. Now this diagram shows the effect of suspending the vesicles in isosmotic sucrose and here we have the effect of suspending them in water. You'll see that there is an initial very large void volume peak in light-scattering material which in the case of this experiment consists of the vesicles themselves coming through in the void volume. And this is followed by the second protein peak with molecular weight of about 50 to 80,000 which represents soluble protein in the vesicle pellet.

In this run, which was done after the vesicles had been suspended in water, we again have a large void volume peak consisting of the broken vesicle membranes. We have this second peak, alpha, which corresponds to this peak here which is the soluble protein contaminant, and now we have a second peak, beta, which consists of this protein vesiculin which has been released by the osmotic shock. And from the retention volume of this peak, we can see that it has an apparent molecular weight of about 10,000.

A better way of preparing vesiculin is to dialyse the vesicle peak from the zonal gradient against distilled water and then to freeze-dry the retentate inside the dialysis bag and then to separate this retentate after freeze-drying and re-suspension in water on an agarose 5-M or Sefedex G-200 column. This diagram shows the results of such an experiment. The upper diagram here compares the elution pattern before and after dialysis and freeze-drying of the vesicles. This is the volume of effluent along here, and here is the percentage of protein recovered.

Well, the heavy line here shows the freeze-dried preparation and you will see that there is now a large peak of low molecular weight protein which wasn't present in the vesicles before they were submitted to dialysis and freeze-drying. The result of that run is shown by the dotted line.

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The lower diagram shows a control programme with a soluble protein peak. There does seem to be a small amount of a vesiculin-like protein released which is shown in this shaded portion of the diagram. Again the dotted line is the soluble protein peak as taken from the gradient. The continuous line is the soluble protein after dialysis and freeze-drying and you see essentially that the same elusion pattern is obtained in each case. There may be a little vesiculin released but note that there were contaminating vesicles in this preparation; there were a few vesicles and the vesicle peak here has been reduced, so probably this vesiculin came from vesicles which were contaminating the soluble protein peak. But most of the protein has a higher molecular weight than vesiculin and consists of proteins with molecular weights ranging from 50 to 80,000.

Vesiculin has an intense absorption at 260 nanometres and we believe that it contains a nucleotide. This can be partially removed by gel filtration on Sefedex G-50 or G-10, and the nucleotide-free vesiculin then appears to dimerise forming a molecule of molecular weight of about 20,000.

Now we've also determined the amino acid composition of vesiculin and the results are shown in my next slide. These two columns show the amino acids in order of abundance in the hydrolysate and these two columns show the amount present in the hydrolysate as moles %. Hydrolysis was carried out in the usual way in 6 normal hydrochloric acid at 110 degrees for 20 hours. The most interesting feature of the analysis is the marked preponderance of acidic amino acids: glutamate with 18 moles % and aspartate with 8.1 moles % and there are also quite large amounts of hydroxy amino acids such as serine. This means that the vesiculin molecule could have a strongly acidic character and its acidic character is also shown by its high affinity for basic ion exchange resins and its lack of affinity for acidic resins.

Now vesiculin therefore resembles other proteins such as chromogranin and neurophysin that are concerned with the packaging of pharmacologically active substances in storage granules. Histamine in mass cell granules is also packaged along with an acidic substance, in this case the acidic polysaccharide, heparin. It's quite likely, in fact, that basic transmitters and hormones are always packaged as the

salts of a polyanion. This would help to slow down loss of the transmitter from the storage granule.

One odd feature of the amino acid analysis is the invariable presence of ornithine or at least of an amino acid that chromatographs with ornithine, and the origin of this is not clear at present, but it is interesting that nervous tissue contains considerable amounts of arginase, the enzyme which converts arginine to ornithine. If one works out the number of potentially acidic groups in the molecule in excess of basic groups, this turns out to be 15 per mole of vesiculin. Here are the total acidics, here are the total basic amino acids and the difference of these gives the excess of acidic over basic amino acids. And you see that this is round about 15 per mole of vesiculin and this interestingly enough is just enough to neutralise the charge on the 12 moles of acetylcholine which is associated with 1 mole of vesiculin in the cholinergic vesicle.

The amino acid present in the lowest amount is tyrosine. Assuming that there is only one tyrosine residue in vesiculin, the molecular weight works out very close to 10,000, in good agreement with the values for gel filtration and also for the value obtained in the analytic ultracentrifuge.

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Well, what is the role of vesiculin in the cholinergic vesicle? I've suggested that its function could be to neutralise the charge on the acetylcholine ion. Many people believe that the release of transmitters occurs as a result of a transient fusion of the vesicle membrane with the external membrane and I've diagrammed this in my concluding slide.

The vesiculin might then act as an ion exchange resin. I've shown the negatively charged vesiculin molecule as R neutralised by the acetylcholine cation. And the vesiculin might act as an ion exchange resin quickly dumping acetylcholine in exchange for sodium or calcium present in the extracellular fluid. And this would occur as soon as the vesicle fuses with the external membrane and opens up so that the contents are in communication with the extracellular fluid.

On reconstitution of the vesicle by the fused vesicle pinching off again and returning, being drawn back into the cytoplasm of the nerve terminal, the vesicle would now be a sodium vesicle. It would be loaded with sodium ions. And the sodium would then have to be exchanged for cytoplasmic acetylcholine synthesised by the extra-vesicular choline acetylase. We've seen how the enzyme choline acetylase, which transfers – catalysis the transfer of acetate groups from acetyl co-enzyme A to choline. We've seen that this enzyme is present in the cytosol and I've shown it synthesising acetyl choline in the cytosol and then an exchange taking place across the vesicular membrane, thereby re-charging the vesicle with acetyl choline with the loss of sodium ions.

Now one would probably have to postulate that a carrier system existed within the wall of the vesicle, within the vesicle membrane to facilitate this exchange. And I've shown this diagrammatically here with the carrier having low affinity for sodium and high affinity for acetylcholine when the combining site of the carrier is facing outwards towards the cytoplasm of the cell and then changing over to a configuration in which the affinity for sodium ions is high, and the affinity for acetylcholine ions is low when the combining centre of the carrier is facing towards the vesicle core.

Well, now clearly abnormalities in either the core protein or the carrier system induced by disease processes or by toxins could have profound effects on acetylcholine storage and release, and so on cholinergic mechanisms. Botulism and myasthenia gravis are two conditions in which abnormalities of this sort could exist. And this provides one reason, apart from intellectual curiosity, why my colleagues and I are pressing on with attempts to understand and characterise the constituents of the cholinergic vesicle and try to understand how the acetylcholine is stored and released.

<End credits>