STUDIES OF THE INTRACELLULAR LOCALISATION OF ENZYMES

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In the first studies of tissue metabolism, metabolic reactions in cells and tissues were determined and then quantitative measurements of enzymes evolved. More recently the distribution of enzymes within the cell has been studied. This has been made possible by the production of more sophisticated apparatus. The knowledge of the localisation of enzymes within the cell in relation to its stucture is very important for the understanding of the functioning of the cell.

In 1839 the cell was identified as the fundamental unit and in this year Schleiden and Schwann independently propounded their cell theory. Now it has been shown that the cell is not just a limiting membrane enclosing a blob of cytoplasm, but instead contains a number of highly organised structures which vary between cell type. Although there is no such thing as a typical cell, some order can be found amongst the complexity. Studies of the structure of the cell have been greatly advanced due to the electron microscope, which has a very high resolving power. The instrument's main disadvantage is that electrons can only cross an extremely high vacuum, and it is therefore impossible to examine living cells. They must be killed and completely dehydrated before use, which gives a risk of distortion of the structures.

When it was known that the cell contained smaller structures, scientists wished to know where a particular enzyme was located within the cell. This has been studied by two main methods: (1) microhistochemistry and (2) biochemistry.

Histochemical methods may be regarded as developing from the staining techniques of classical histology since they depend on the liberation of a staining substance in tissue sections as a result of enzyme action, followed by microscopic examination of the stained tissue. In earlier studies the tissue sections were dehydrated by treatment with acetone and then embedded in warm paraffin wax. This denatures most enzymes, so recently tissues are quickly frozen and sectioned at low temperatures. There are certain limitations on the histochemical approach which are as follows:

(1) The product of the enzyme reaction must stay at the site of the reaction, so a fixing substance may be necessary.

(2) A staining compound is necessary, but must not interfere with the reaction.

(3) The fixing and staining compounds must be able to enter the cell freely without disrupting the integrity of its structure.

Because of these limitations the method is only useful for a few enzymes. An example of such an enzyme is acid phosphatase. A lead trapping agent is used which combines with the phosphate ions liberated in the reaction to give lead phosphate, an electron dense compound which will show up under the electron microscope.

The biochemical approach consists of isolating the enzyme and then studying the reaction it catalyses. The first stage is to obtain a cell-free homogenate where the cell membrane has been disrupted but the membranes around the intracellular particles remain intact. The procedure for this varies for different cell types, so a standard method has been developed for each. There are several ways of disrupting the cell membrane including (1) stress by velocity gradients, (2) alternate freezing and thawing, (3) enzyme action, and (4) sonication. Method (1) can be standardised most easily and is the preferred method. Methods (2) and (3) are harder to standardise and more random in their action.

The best method involving stress by velocity gradients is by use of a rotating pestle and mortar. An example of this is the Potter-Elvehjem homogenizer in which the mortar is moved up and down by the operator, past a rotating cylindrical pestle. The maximum rate of shear is at the point of contact with the liquid and rotating pestle, which will distort the cell membrane and so disrupt it. The disadvantage of this homogenizer is overheating which could destroy an enzyme by denaturation of its protein structure. The Potter-Elvehjem homogenizer is however most commonly used. The procedure can be standardised by the number of strokes and the speed of the pestle's rotation.

The medium used for the homogenate has a profound effect on the cytological and biochemical properties of the particulate components. Those of the mitochondria in different media have been studied most thoroughly and have therefore served as a basis for the selection of media.

It is very important that during homogenization the intracellular membranes should remain intact. There are several methods for checking this. One should get reproducible results for the distribution of enzymes within the cell. When an enzyme is located within a cellular particle, its activity will be latent, i.e. it will only appear after the membrane around this particle has been disrupted. Therefore if the homogenate is a good one, these enzymes

should display latency. Fractions of the cell can be obtained by differential centrifugation. The distribution of several enzymes in these fractions is known, so that if the homogenate is good, the distribution pattern will be identical to the standard. If however the membranes have been disrupted within the cell, this distribution will alter, the activity probably being found mainly in the cell supernatant.

Obtaining these cell fractions, which I have mentioned above, is the next step in the procedure for the localisation of enzyme activity. The various structural components of a homogenate sediment in the centrifuge at different rates, primarily due to their difference in size, and can be separated by a series of spins usually at increasing speeds. By resuspending the centrifuged pellets and centrifuging again, a number of fairly homogenous fractions can be obtained. This method is termed differential centrifugation.

As a result of the work by Schneider, Claude, Hogeboom, Dounce and others a more or less standard fractionation scheme has been adopted to give the following fractions: (1) intact cells and tissue debris, (2) nuclei, (3) mitochondria, (4) lysosomes, (5) microsomal fraction, (6) supernatant.

Many enzymes can be studied by this method, several on the same preparations thus making comparisons of activity and requirements easier. The fractionation scheme must vary with different tissues because of the different cell structures, and now schemes have been devised for nearly all tissues from rat liver cells to bone.

There are however several disadvantages in this method. Separation from its natural environment may affect the enzyme. Fractions may be contaminated by particles from another fraction. In addition the enzymes in a fraction are not necessarily all present in the same kind of particle. For example, De Duve found that the mitochondrial fraction contained lysosomes. He was studying the distribution of alkaline phosphatase activity and found that the total activity of the cell fractions was less than that of the homogenate. On leaving in a refrigerator overnight, the activity increased in the mitochondrial fraction so that the total activity equalled that of the homogenate. Careful differential centrifugation separated these particles, the lysosomes being smaller than the mitochondria sedimented more slowly. It was much later that cytologists were able to identify these structures under the electron microscope. Mitochondria seem to be homogenous with respect to size and enzymic composition, but lysosomes although enzymatically homogenous have a wide range of size, and this makes it difficult to obtain them free from both mitochondria and microsomes and *vice versa*.

Another example where more than one structure may be present within a fraction is in the microsomal fraction, which contains fragments of endoplasmic reticulum either rough or smooth. The rough membrane has ribosomes on its surface which are the site of incorporation of amino acids to form proteins. This fraction also contains vesicular elements coming from the Golgi, plasma, and nuclear membranes.

To conclude the disadvantages of the homogenate technique, one may mention that when there are many cell types present in the homogenate, the enzymic activity cannot be associated with a particular cell type as in the histochemical method, where the cell can be identified under the microscope.

To obtain a complete picture of the localisation of enzymic activity, each fraction is analysed by sedimenting in a density gradient which includes the value of particle density associated with the enzyme. After equilibrium is reached particles rest in a band centered round their own buoyant density. Each layer is then analysed for enzymic activity.

The usual swing-out heads used in centrifuges introduce errors by disturbing the density gradient on swinging up to the horizontal when gathering speed, and down again to the vertical at the end of the spin. This is eliminated by a new type of centrifuge using the method of zonal centrifugation, whereby the density gradient and fraction under test can be introduced and removed whilst the centrifuge head is still spinning horizontally.

Interpretation of results requires more care. Morphological and biochemical properties are assigned to the fractions bearing in mind the components of the original homogenate and the fractionation techniques orginally employed. These components of a homogenate may finally be equated with certain intracellular entities.

It is important to show that the sum activity of all fractions equals the activity of the unfractionated homogenate. The possibility that inhibitors or activators may be present must be considered. In such a case the distribution of enzyme and of inhibitor or activator may be entirely different, with the result that the sum of activities of the fractions will be greater or less than the activity of the homogenate. It is then necessary to measure the activities of the fractions in all permutations and combinations, as well as separately, in order to determine the localisation of the inhibitor or activator as well as to eliminate the possibility of denaturation during the isolation procedure. When these

requirements have been met, the localisation of an enzyme in a cellular component is indicated in three ways: (1) a large percentage of the total activity of the homogenate is in that fraction; (2) the specific activity of the fraction is several times greater than that of the homogenate; (3) the specific activity of the fraction remains constant upon repeated sedimentation. These criteria have been satisfied for only a few enzymes.

The identification of the subcellular fractions can be performed either by examining under an electron microscope, which if the fraction has been carefully prepared should show only one type of structure, or by enzyme assay with an indicator enzyme whose distribution is well known.

De Duve has pointed out the two fundamental assumptions in biochemical studies of the intracellular distribution of enzymes. They are that an enzyme is localised in one intracellular site, and that populations of subcellular fractions are enzymatically homogenous. Some untenable assumptions may be mentioned, for instance, that analogous particles in different cells, tissues or organisms should have the same size, shape or destiny and would therefore be found in the same fraction. Because this cannot be assumed, the fractionation scheme employed depends on the tissue concerned. In like manner it cannot be assumed that the intracellular localisation and certain properties of a particular enzyme such as latency remain invariant from cell to cell. This would have to be established for each application.

The absence of a certain activity from a certain fraction does not necessarily mean that the intracellular component does not possess the enzyme because the activity may have been detached or liberated during fractionation. Many nuclear and some mitochondrial and lysosomal activites are of this kind. Conversely, the presence of an activity in a fraction does not guarantee its original association with a particle *in situ*. The enzyme may have originally been soluble or detached from one particle type, only to become attached to a second particle type. For example, the attachment of basic proteins such as cytochrome c to ribonucleic acid of ribosomes is known to occur.

One of the most striking results from studies of the intracellular localisation of enzymes is that in many important metabolic systems all the enzymes and coenzymes are present in the same particle. For example, all the components of the oxidative chain are found in intramitochondrial particles. This however has been overstressed in the past, for only two of the enzymes of the tricarboxylic acid cycle are found entirely in the mitochondria. The rest are mainly localised in other fractions.

As I have shown, there are many pitfalls in the interpretation of results, but with care the biochemical method is very useful in conjunction with cytology for advancing our knowledge of the intracellular distribution of enzymes.

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