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COMMENTARY

THE INFLUENCE OF TISSUE ENVIRONMENT ON THE RATES OF METABOLIC PROCESSES AND THE PROPERTIES OF ENZYMES

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If conclusions concerning the properties of enzymes *in vivo* are to be drawn from those determined *in vitro* it is important to ascertain whether the properties in whole organs differ from those in purified preparations *in vitro*. The purpose of this brief and necessarily incomplete review is to compare and contrast the properties of purified enzyme preparations *in vitro* with the behaviour of those enzymes studied in whole organs and thereby to emphasize the influence of tissue environment on the rates of metabolic processes and the properties of enzymes.

Determination of the intracellular distribution of enzymes, in tissues has revealed within the cell a catalytic organisation [1] which parallels the cell morphology. The subcellular organelles, many of which have specific metabolic functions, are arranged in a structural pattern, and in addition there may be numerous metabolic compartments and diffusion barriers. At a more gross level of organization the cells are arranged within organs in close juxtaposition with other cell species, blood vessels and extracellular fluid, each component making up the structural and metabolic whole.

The study of metabolic pathways and rate controlling enzymes within those pathways involves the isolation of subcellular particles, solubilization and purification of enzymes and use of tissue extracts, slices or homogenates. The preparation of these experimental systems involves destruction of the catalytic organization but the properties of enzymes and metabolic pathways as determined using such systems are often directly applied to discussion of the control of those processes in intact organs *in vivo*.

Results obtained using isolated perfused organs have revealed differences in the rates of metabolism and properties of enzymes as measured in whole organs when compared with those obtained using other preparations. The K_m value obtained using highly purified enzymes is used to provide information as to the efficiency with which an enzyme will function at the steady-state substrate concentrations which it normally encounters in the cell. A similar approach can be made using isolated perfused organs where, unlike *in vitro* experiments, the cellular and subcellular structures and permeability barriers which may influence the accessibility of the substrate to the enzyme are retained. Thus, it is possible to calculate the initial concentration of substrates giving half maximum rates of removal or product formation in the isolated perfused organ. This quantity can be called the "apparent K_m" of the particular enzyme. It should, however, be noted that an isolated perfused organ system, although it approaches nearer to the *in vivo* situation, is not ideal in that perfusion rates and patterns of perfusate flow through the tissue may not be physiological and in addition the tissues are isolated from neurological and hormonal influences. Even if the animals' own blood is used as a perfusion fluid with addition of heparin to avoid coagulation, and sometimes of PGE_1 which inhibits platelet aggregation this might affect the physiological condition of the organ.

What are the factors one should consider which might be the cause of the properties of purified enzyme being inapplicable to isolated cells, whole organs, or the *in vivo* situation?

1. THE INFLUENCE OF THE TYPE OF TISSUE PREPARATION ON THE RATES OF A TOTAL METABOLIC PATHWAY

The influence of the type of preparation on the rates of metabolic processes can be shown by hepatic gluconeogenesis, glycolysis and ketogenesis. In liver slices the rates of these processes (Table 1) are much lower than those measured in the intact isolated perfused liver. In addition the rate of glycolysis in fortified liver homogenates [2] and in slices [3, 4] rapidly decreases as incubation progresses, whereas in isolated perfused liver the rate is linear with time until the substrate has been depleted. A preparation at a different state of structural preservation is that of isolated intact liver cells [5]. The rates of gluconeogenesis and ketogenesis in this preparation are comparable with those measured in the perfused liver [6].

The differences between liver slices and other preparations may result from the damage sustained by the tissue during its preparation. Slices contain a proportion of damaged cells and thus enzymes, cofactors and inorganic ions can leak out into the incubation medium. The smaller rate of gluconeogenesis, an

Table 1. The rates of metabolic processes in rat liver slices and the isolated perfused rat liver

Metabolic process	Substrate	Metabolic rates		Ref.
		slices	perfused liver	
Gluconeogenesis	lactate (10 mM)	0·55	1·06	11
μmoles/min/g	alanine (10 mM)	0·23	0·66	
Glycolysis	glucose	0·7	2·47	16
μmoles/min/g				
Ketogenesis	oleate (2 mM)	5·0	68·0	17
μmol/h/g	butyrate (2 mM)	16·0	64·0	

energy requiring process, is probably due in part to the rapid irreversible fall in the adenine nucleotide content of liver slices which occurs as incubation progresses [6]. This fall does not occur during incubation of isolated intact liver cells or liver perfusion [6, 7].

These examples are drawn from intermediary metabolism. There are indications that the same principles apply to the study of drug metabolism in liver. Von Bahr *et al.* [8] used the isolated perfused rat liver to compare the rates of removal of a number of drugs in this system with the rates measured *in vivo*. These workers found that there was a close agreement between the half lives *in vivo* and in the perfused liver for drugs with a small volume of distribution such as antipyrine and phenylbutazone. In contrast, drugs such as nortryptyline and desmethylimipramine which have a large volume of distribution, were removed some thirty times more rapidly in the perfused liver when compared with the rates *in vivo*.

Although the rate of metabolism of a drug in the isolated liver may be similar to that *in vivo*, the relative amounts of drug metabolised by alternative pathways may differ in the two experimental situations. The total rate of metabolism of imipramine in perfused rat liver [1] is very similar to that measured using rat liver microsomes *in vitro* [10] while in the perfused organ there is decreased demethylation and increased hydroxylation.

2. INFLUENCE OF TYPE OF TISSUE PREPARATION ON THE RATE OF INDIVIDUAL CATALYTIC STEPS AND ENZYME PROPERTIES INVOLVED

Evidence exists to show that the removal of an enzyme from its natural environment alters its properties. Xylitol is converted to glucose in rat liver [11, 12], the first step being via the NAD-linked cytoplasmic polyol dehydrogenase [13]. In the isolated perfused rat liver xylitol removal is rapid, the rates of removal rising with the initial xylitol concentration [14]. The initial concentration of xylitol giving half maximal rates of removal ("apparent K_m") in perfused liver was 3·2 mM. This value is considerably higher than the K_m for purified NAD-linked cytoplasmic polyol dehydrogenase of sheep liver (0·18 mM at pH 9·6) [15] and that of the guinea-pig liver (0·6 mM at pH 8·1) [13]. These results suggest that in the whole liver the properties of polyol dehydrogenase differ from those determined using the purified enzyme removed from its normal environment and that the optimal rates of xylitol removal only occur at concentration well above the K_m as determined *in vitro*.

In other cases although the K_m as determined *in vitro* and the "apparent K_m" determined in intact whole organ may be identical, other properties of the enzyme reaction may be modified. This is illustrated by the monoamine oxidase reaction in liver when kynuramine is the substrate [18]. The "apparent K_m" for product formation (4-hydroxy-quinoline) was 0·07 mM which is very close to the K_m (0·05 mM) measured *in vitro* using the same substrate [19, 20]. The fact that the K_m of the purified enzyme and that determined in the whole organ are similar would suggest that within the liver there is no compartmentation of this substrate. However, inhibition by high substrate concentrations appears to be a common phenomenon among double-displacement-type enzyme systems *in vitro* and has often been observed with monoamine oxidase when using kynuramine, benzylamine or dopamine as substrates [20–23, 39]. With kynuramine, this is probably an example of substrate inhibition in that the product of the reaction, 4-hydroxyquinoline, is not inhibitory even at high concentrations *in vitro* (5 mM) [23].

In the perfused rat liver, the rate of oxidative deamination of kynuramine rises with substrate concentration. But, the substrate inhibition seen *in vitro* does not occur, even when the substrate concentration is raised as high as 4 mM in the perfusion medium. Why the enzyme is inhibited *in vitro* and not *in vivo* is not known. One possibility could be that *in vitro* the aldehyde generated by reaction of monoamine oxidase could react irreversibly in a Schiff base reaction with the enzyme.

When preparations of benzylamine (free base) in aqueous solution are used for assay of monoamine oxidase, high substrate concentrations are inhibitory. However, with benzylamine hydrochloride as substrate, no such inhibition occurs [24]. The cause of this substrate inhibition when the free base is the substrate has been found to be aldehyde contamination of the substrate [24]. It seems unlikely, however, that the marked sensitivity of this enzyme to aldehyde inhibition will have any significant effect *in vivo*, since the

NAD^+-linked aldehyde dehydrogenase has a very low K_m value for aromatic aldehydes (see ref. 24). Thus, the possibility that chemicals are contaminated with products which inhibit enzyme systems should be considered in the interpretation of results.

What are the mechanisms by which differences in enzyme properties occur? Some of the differences may be related to the presence of diffusion barriers and metabolic compartments in intact whole organs which are destroyed during enzyme preparation and purification procedures. Another possibility concerns the more intimate environment of the enzyme, namely either its attachment to subcellular particles or its interaction with other enzymes in the same or other metabolic pathways.

Cellular membranes contain phospholipid, but the type of phospholipid varies in different organs, organelles and species [25, 26]. Little is known about the significance of these variations and no correlation of membrane function with phospholipid composition has emerged. Many enzymes are located in membranous structure within the cell, and until recently some of these enzymes were thought to be resistant to solubilization and purification. It is now apparent that many membrane bound enzymes require the lipid components of the membrane for activity [27–29]. Pig liver monoamine oxidase has an extremely high affinity for cardiolipin [30] and the studies of Oreland and Ekstedt [31] have shown that this enzyme depends on the associated lipid for its conformational stability. The effect of removing phospholipids on the activities of membrane enzymes may be found in the studies of Mavis *et al.* [32] and Sanderman [33]. The latter author for example, has found it possible to reactivate the membrane bound enzyme, C_{55}-isoprenoid alcohol dehydrogenase of *Escherichia coli* by adding lecithin. The properties of the enzyme depended upon the type of lecithin added.

Let us consider the environment of monoamine oxidase within the cell of which something is known. It is firmly attached to the outer mitochondrial membrane [34]. Multiple forms of monoamine oxidase as separated by gel electrophoresis [35] are attached to varying amounts of phospholipid membrane material [36, 37] and possess different kinetic properties, substrate and inhibitor specificities *in vitro*. The procedures for solubilizing the enzyme are vigorous and harsh [38, 39]. The membrane environment conveys certain allotopic properties to the bound enzyme [37]. The observation [37] that no separable bands of monoamine oxidase activity can be obtained when the rat liver enzyme is subjected to treatment with chaotropic reagents (presumably by removing the phospholipid membraneous material) emphasized the importance of this aspect of enzyme environment and its contribution to multiplicity. It has been suggested that the same might hold true *in vivo* [19]. The recent observation that liver and brain mitochondria are heterogeneous with regards to monoamine oxidase activity when using a number of substrates is significant in this respect [40, 41].

Interaction with membrane and lipid material has been shown to modify the kinetic properties of other enzymes [42]. Hexokinase is reversibly bound to mitochondria in ascites tumour cells [43] and pig heart muscle [44] and the properties of hexokinase change when it is removed from its particulate environment. Thus Hernandez and Crane [45] showed that about 25 per cent of skeletal muscle hexokinase was bound to sarcoplasmic vesicles and this particulate enzyme has a K_m for adenosine 5′-triphosphate (ATP) of 0·28 mM as compared with 1·23 mM for the soluble enzyme.

In the brain a proportion of tryptophan hydroxylase is found to be associated with the synaptosomes and this has been suggested as the rate-limiting step in the synthesis of 5-hydroxytryptamine (5-HT) [46]. The K_m of tryptophan hydroxylase varies considerably depending on the type of preparation used (Table 2). The homogenate and the particulate (synaptosomal) enzymes have a K_m of 15–20 μM using the synthetic cofactor $DMPH_4$ (2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine) (see Table 2). However, using the same cofactor and a partially purified lysed or sonicated synaptosomalenzyme, a K_m of 250–400 μM is observed. However, with $DMPH_4$ as cofactor, inhibition of the homogenate or synaptosomal enzyme acti-

Table 2. The K_m of tryptophan hydroxylase measured in various preparations are listed

Preparation	Cofactor*	K_m (μM)	Ref.
Guinea-pig stem; homogenate	$DMPH_4$	20	48
Guinea-pig brain stem; partially purified	$DMPH_4$	250	48
Rat brain	$DMPH_4$	300	46
Rat brain stem; homogenate	$DMPH_4$	30	49
Rat brain stem; partially purified	BH_4	5	49
Pig brain stem particulate	$DMPH_4$	15	47
Pig brain stem particulate; purified	$DMPH_4$	400	47
Rabbit hindbrain	$DMPH_4$	290	50
Rabbit hindbrain; partially purified	BH_4	50	50
	6-MPH_4	78	50

* $DMPH_4$: 2-amino-4-hydroxy-6,7 dimethyltetrahydropteridine; BH_4; tetrahydrobiopterin; 6-MPH_4: 2-amino-4 hydroxy-6-methyltetrahydropteridine.

vity occurs when compared with a preparation where no cofactor is used. This inhibition does not occur with a partially purified enzyme: under these conditions there is an increase in V_{max}[46–50]. But studies [49, 50] using BH_4 (tetrahydrobiopterin) a naturally occurring cofactor, have shown that the K_m for the partially purified enzyme preparation is lower than that when $DMPH_4$ is present (see Table 2) and the V_{max} is higher. Another property of tryptophan hydroxylase which is altered by changing the cofactor is its sensitivity to inhibition by high concentration of its amino acid substrate *in vitro*. This inhibition is seen only with BH_4 (above 0·2 mM tryptophan) as the cofactor, but not with $DMPH_4$ at concentrations of tryptophan above 0·2 mM. It has been inferred from these studies that inhibition of brain tryptophan hydroxylase by high tryptophan levels can occur *in vivo* [50] resulting in the decreased synthesis of 5-HT. The variation of 5-HT accumulation as a function of tryptophan concentration was described by a hyperbolic curve [51]. This has been interpreted as showing inhibition of 5-HT accumulation by tryptophan [51]. If indeed this was a correct interpretation it might then alternatively be explained on the basis of product or end product inhibition [52] or substrate inhibition [50]. The relevant *in vivo* studies with high concentration of tryptophan and the measurement of the direct product (5-hydroxytryptophan) has not shown substrate inhibition [53]. Many of the conflicting data reported on the kinetic properties of tryptophan hydroxylase may be explained on the basis of cofactor requirement of the enzyme since the nature of the cofactor has not been identified. In addition the substrate inhibition of tyrosine hydroxylase and hydroxylation of phenylalanine by this enzyme are also pertinent to the present commentary. It is reported that *in vitro* inhibition of tyrosine hydroxylase by its substrate (tyrosine) occurs [54, 55]. However, in an isolated preparation of vas deferens [56] and *in vivo* studies [17] substrate inhibition does not occur when tyrosine levels are raised several times above the K_m for tyrosine. These results tend to cast doubt on the physiological significance of the *in vitro* work and may indirectly provide evidence for the importance of permeability barriers in the transport of tyrosine into cells where the enzyme is present.

It should be emphasized that the enzymes discussed above are assayed *in vitro* under conditions far removed from those existing *in vivo*. Attempts must be made to approximate *in vitro* the features of the environment *in vivo*. In discussing the properties of lactate dehydrogenase (LDH) isoenzymes and their distribution in tissues, Vesell [58] has shown that kinetic measurements are substantially different when they are obtained under more physiological conditions. Marked differences in sensitivity between isolated purified human LDH_1 and LDH_5 are observed [58]. The latter isoenzyme is not inhibited by high pyruvate concentrations as is LDH_1. Thus LDH_5 in anaerobic tissue such as skeletal muscle may be functional despite pyruvate concentrations in this tissue which might inhibit LDH_1. On the other hand, LDH_1 is predominant in tissues with relatively low concentrations of pyruvate such as brain and heart. However, pyruvate and lactate concentrations high enough on the basis of results obtained *in vitro* studies to inhibit LDH_1 do not occur *in vivo* [59, 60]. At most, they are below 1 mM in the case of pyruvate and 25 mM for lactate even under extremely anaerobic conditions [58, 61]. Under most conditions the LDH in the cell is in great excess over the substrate [62]. Even under the anaerobic conditions of the Novikoff hepatoma, where rat liver LDH activity is decreased but lactate production increased, LDH has been reported to be in 300-fold excess over substrate [62].

Some of the examples cited above are those in which enzyme properties have been shown to be modified by attachment of the enzyme to structural components of the cell. Other mechanisms exist whereby the metabolic control may be effected through interaction between enzymes and other macromolecules within the cell. Lehninger [63] suggested that protein–protein interactions of an allosteric nature may occur within the cell and thus influence metabolic control. Interactions of this type have been described for a number of pairs of enzymes *in vitro*. Rabbit muscle aldolase activity is increased in the presence of glyceraldehyde-3-phosphate dehydrogenase under conditions where the latter is not enzymically active [64]. Conformational changes have been suggested to explain the changes in properties of glutamic-pyruvic-transaminase in the presence of phosphorylase [65] and Pogell *et al* [66] have described the reversal and prevention of the adenosine 5′-monophosphate (AMP) inhibition of fructose 1–6 diphosphatase in the presence of phosphofructokinase.

In the intact animal, certain organs can synthesize pharmacologically active agents by enzymatic processes. In some cases the failure to demonstrate such reactions *in vitro* has been attributed either to low level of enzyme activity or the presence of a natural inhibitor in the tissue. Inhibitors may be present in a bound inactive form in undisturbed tissues and may be completely or partially released and activated during tissue extraction or homogenization. To cite two examples, natural inhibitors have been described for the enzymes tryptophan hydroxylase and dopamine β-hydroxylase (DBH). Whether such an inhibitor exerts a physiological role in regulating the activity of tryptophan hydroxylase and thereby the level of 5-HT in tissues, is not known and must wait further characterization of the inhibitor. However the intestinal tract makes a significant amount of 5-HT *in vivo*. The lack of demonstrating this reaction *in vitro* may result from low levels of enzyme activity or from the presence of a natural inhibitor in this tissue [67, 68]. More is known about the natural inhibitor of copper dependent dopamine β-hydroxylase (DBH) which catalyzed the β-hydroxylation of dopamine to noradrenaline [69–71]. Although purified preparations of this enzyme have shown acti-

vity, whole homogenates of animal tissues have little or no DBH activity [72–74]. One of the major difficulties encountered in assaying DBH activity in tissues is the presence of endogenous sulphydryl inhibitors [72–74a]. These inhibitors are thought to chelate the copper which is an integral part of the enzyme molecule [72, 73] and therefore during assay of this enzyme Cu^{2+} is added to prevent this inhibition. Cu^{2+} ions presumably neutralize the sulphydryl inhibitors which probably appear during extraction procedures as a result of enzyme action (proteinases, glutathione reductase) or protein denaturation. In the presence of molecular oxygen the concentration of these sulphydryl groups progressively falls. There is no demonstration that *in vivo* these sulphydryl inhibitors are active on DBH. The question arises as to whether they are normally in contact with the enzyme. Conversely, natural inhibitors may be neutralized during manipulation of the tissues.

An additional factor is the accessibility of substrate to the enzyme. For example, angiotensin II is not inactivated during passage through the human lung [75]. Lung homogenates do however rapidly inactivate this peptide [76]. Carboxypeptidease-N is present in rat lung tissue preparations [77] but this enzyme does not inactivate bradykinin in the isolated perfused lung [78]. It can be concluded from these results that under normal conditions there is no contact between these peptides and the microsomal peptidases in intact lung whereas when the tissue is disrupted enzyme and substrate can come into contact. Alternatively, a natural inhibitor may be present in the intact tissue.

Metabolic utilization can be restricted by limitation of substrate uptake by permeability barriers. Glutamate is rapidly utilized in rat liver homogenates and mitochondria (Table 3), whereas in preparations in which there are intact cells, namely liver slices and the isolated perfused liver, the rates of metabolism are much lower. The intact cells thus provide an effective barrier to glutamate uptake, which is not present in homogenates or isolated mitochondria. Similar observations have been made for malate and succinate. [11, 79].

A further question which arises out of the observations discussed above is whether it is possible to demonstrate in the intact mammal the mechanisms of inhibition by drugs observed *in vitro* using tissue preparations.

There is evidence that the action of a drug determined *in vitro* may not be observed *in vivo*. Prenylamine (Segontin) inhibits purified human placental monoamine oxidase *in vitro* [80]. *In vivo* experiments with prenylamine in rats did not reveal any inhibition of monoamine oxidase [80]. This might be expected since it is known that compounds carrying a methyl group in the α-position of the nitrogen atom are weak reversible inhibitors of monoamine oxidase *in vitro*.

Table 3. Glutamate metabolism in rat liver preparations

Preparation	Rate of metabolism (μmoles/min/g)	Ref.
Homogenate	1·4	89
Slice	0·01	89
Perfused liver	0·15	11

In man, data has been provided which shows that in some respects prenylamine has action similar to that of reserpine [82]. Catecholamines liberated by these drugs from bound stores are predominantly degraded by monoamine oxidase in tissues to the deaminated products. This show itself in a predominance of oxidatively deaminated, over *o*-methylated metabolites in the urine [82–84]. The ratio of urinary *o*-methylated to deaminated products does not provide any support for an *in vivo* monoamine oxidase inhibition by prenylamine; otherwise an increase in metadrenalines and a decrease in oxidatively deaminated metabolites would be expected [82, 84].

In contrast, the inhibitory action of the anti-inflammatory drugs—indomethacin, aspirin and salicylate—on tissue prostaglandin synthetase activity *in vitro* [85] probably occurs in man taking therapeutic doses of these drugs. Hamberg [86] found a marked lowering of the urinary excretion of the major PGE_1 and PGE_2 metabolites after treatment with these drugs in man.

CONCLUSIONS

The subject matter of this paper concerns the ways in which the rates of metabolic processes and the properties of enzymes can be influenced by the type of tissue preparation used. The results of experiments using purified enzyme tissue extracts and slices have to be interpreted with caution because when these techniques are used the resultant system is artifical [87].

The preservation of the structural integrity of an organ at the gross and microscopic levels together with such factors as permeability barriers and metabolic and natural inhibitors is of great importance in the study of the control of enzyme activity. Thus extrapolation from kinetic data derived under optimal conditions *in vitro* to conditions *in vivo* where entirely different concentrations of enzyme, coenzyme and substrate may exist, should be performed bearing in mind these considerations.

A pressing problem in clinical biochemical pharmacology is the development of a reliable methods to show that the mechanisms of drug action, drug activation and inactivation observed using pure enzymes, tissue extracts, tissue slices, cells in culture or intact perfused organs occur in the normal intact organism. Such methods, if they measure the mechanism through which a drug exerts its therapeutic actions, would provide the physician with a way in which therapy could be used to greater effect.

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