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Techniques and applications of extracellular space determination in mammalian tissues

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Summary. This review summarizes the ways in which the extracellular space (ECS) may be estimated in mammalian tissues, and briefly describes some of the uses to which the EC confinement of certain molecules (markers or tracers) may be put in the elucidation of physiological functions.

The introductory section is followed by a description of the more commonly used marker molecules and their functional characteristics, and of factors likely to lead to the spurious over- or under-estimation of the ECS. Certain alternative methods are also described, in particular those based on morphological and electrical criteria which seek to demonstrate small, functionally important, changes in the size of specialized regions of the ECS (e.g. lateral cellular interspaces) without necessarily being required to provide a quantitatively precise estimate of their size. Section III describes the results of measurements of ECS in various mammalian tissues (muscle, gastro-intestinal tract, nervous tissue, crystalline lens, placenta, lung and kidney) and some applications of EC markers to investigation of cellular function (e.g. uptake of metabolic substrates and epithelial transport) and, in outline, characterization of capillary permeability.

The available literature in this field is very extensive, and in the interests of brevity the reader is, where appropriate, referred to previous reviews covering specialized aspects of ECS determination and related topics.

I. *What is the extracellular space and why measure it?*

The extracellular space (ECS), as its name implies, is the fluid compartment situated externally to the cells of the body. It comprises the plasma component of the vascular space, and the interstitial fluid. It is the arithmetic sum of these two compartments which is usually implied when the ECS is referred to.

The volume of the ECS within a tissue may conveniently be assessed as the volume of distribution at equilibrium concentration, or by following the uptake or washout kinetics, of a marker or tracer molecule introduced into the tissue (e.g. by infusion) and unable to penetrate the intracellular (IC) fluid. These and certain alternative techniques for ECS determination will be considered in detail in section II. Infusion to equilibrium concentration may also be applied to the measurement of plasma volume, using a marker molecule which only slowly effluxes from the capillary bed, e.g. labelled serum albumin or polyvinylpyrrolidone (see for example Law[107,114]. The size of the interstitital space (ISS) can be calculated by subtraction of the plasma volume from the entire ECS (see for example Daniel et al.[47] and Law[115]). Certain specialized techniques have been developed for examination for ISS fluid, e.g. tissue centrifugation[117] or direct microsampling using multifilamentous nylon wicks[6,7,169], and recently a method has been evolved for the measurement of ISS volume changes using extracellularly implanted ion-sensitive micro-electrodes[81].

The brief description of the ECS given above, although adequate for many experimental purposes, is nevertheless an over-simplification. Firstly, the ISS is not a static pool: the capacity of a marker molecule to achieve equilibrium concentration within a given organ will be influenced by the rate of fluid draining from that organ either in the form of lymph or as cerebrospinal fluid (CSF) (III, C1), bile (III, B6), etc. Secondly, there are certain relatively inaccessible regions of the ECS in which equilibrium may be unacceptably slow. It has long been recognized that fluid associated with dense connective tissue falls within this category[148] and its presence poses certain problems of ECS determination[27,73]. Relatively impe-

netrable regions include also the sarcoplasmic reticulum and other IC microtubules within muscular tissue[14,59,65,92,161,171], saculae and caveolae on the surface of smooth muscle cells[64,65], and possibly also the microtubules in cerebral tissue[38]. Such regions may be accessible to some markers but not others[59]. They are small but may be important in terms of a tissue's response to changes in the character of its environment (e.g. the EC nature of the sarcoplasmic reticulum in skeletal muscle may explain the osmometric behaviour of muscle cells[14,129]). EC regions of major physiological significance lie between the cells of certain transporting epithelia (e.g. in kidney and intestine). Functionally important changes in the width of these paracellular pathways may occur too rapidly, or be too small, to be estimated by conventional marker technique, and morphological or electrical methods must be employed. Lastly, it is undoubtedly true that many - possible all - supposedly EC marker molecules exhibit some degree of IC penetration, although it may be possible to allow for this by following the fast (EC) and slow (IC) phases of their uptake or efflux from tissues.

Before passing to more detailed consideration of the problems of ECS measurement it is necessary to stress why such measurements are considered so important. In its most straightforward application, the volume (and composition) of the ECS must be determined if a reliable estimate of the distribution of solutes between EC and IC compartments it to be obtained from analysis of the whole tissues. Many pioneering studies on ECS volume were performed with this end in view, although to some extent this approach may be said to have been superseded by the development of ion-sensitive IC electrodes. The special case of the ECS in transporting epithelia has already been referred to. For many experimental purposes it is the EC confinement of a marker, rather than a knowledge of its precise volume of distribution (although this may also be determinable) which is the crucial factor: this characteristic has found extensive application in the paired or multiple tracer-dilution technique and in the investigation of capillary permeability (see section III).

This review is confined to results obtained using EC markers in mammalian tissues.

II. *Methods of measuring the extracellular space*

The most commonly used, widely applicable, and potentially accurate method of measurement of the ECS involves, as has already been briefly outlined, the introduction of marker molecule into the space and determination of its concentration at equilibrium. The requisite characteristics for such a marker may be summarized as follows: it should a) readily and uniformly distribute throughout the entire anatomical ECS, b) be excluded from the cells, c) exert no influence on the size of the ECS, d) not be metabolized, e) be of uniform molecular size and diffusibility (i.e. not be poly-disperse), f) be easily and accurately estimated at low concentrations - at the present time this almost invariably involves the use of radioisotopic labeling. Lastly, any tendency for the marker to be lost, e.g. by urinary elimination following whole-body infusion, or by lymphatic draining, should be minimal or, if this is impracticable, allowed for when determining the attainment of equilibrium conditions (urinary loss may be prevented by bilateral nephrectomy).

Following introduction of marker by whole body infusion, (e.g. i.v. until a constant plasma concentration is achieved) individual organs may be excised and their ECS calculated from their marker content. Alternatively, ECS of individual organs can be estimated in situ by a non-destructive method involving comparison of the mean transit times of rapidly injected boluses of suitable vascular and EC markers (for the theory of this technique see Chinard[34], Martín de Julián and Yudilevich[136] and Zierler[210]). In vitro methods may be used in which tissue slices (e.g. brain, liver, kidney), sheets or strips (e.g. diaphragm, intestinal, gall bladder and vascular walls) or whole organs (e.g. isolated cervical ganglia, small skeletal muscles) are allowed to achieve equilibrium during incubation in appropriate physiological solutions containing markers. Recently Hansen and Olsen have developed an ingenious technique whereby the extent of the interstitial component of the ECS of the cerebral cortex, which is affected by and therefore reflects changes in cell volume, can be detected through variation in ionic ECS marker concentration as sensed by implanted ion-sensitive micro-electrodes[81]. The advantage of this method lies in its capacity to provide instantaneous information which could not be obtained by conventional techniques. Its potential drawback is its dependence upon a significant difference between the time constants for changes in cell volume and 'escape' of the marker either into the cells themselves or back into the vascular bed. In cerebral tissue these appear to be suitably different[81], but the technique has not yet been applied to other tissues.

A wide range of molecules have, with varying degrees of justification, been regarded as satisfying the requirements listed above. The two principle categories comprise saccharides and ions. Among the former, inulin (which is readily available as the ^{3}H-methoxy- or ^{14}C-carboxylic acid compound) has been widely favored. Labeled forms of sorbitol, mannitol, sucrose and raffinose have also been extensively used. Other studies have employed arabinose[9], erythritol[53], dulcitol[173] and methyl-D-glucosides[77]. In recent years L-glucose has proved a valuable ECS marker in experiments investigating uptake of metabolites by

brain[207], pancreas[48] and placenta[54,118,204-206], but it is believed to be taken up by facilitated diffusion by intestinal[146] and salivary gland cells[209]. It may also penetrate cells in the liver[77], renal medulla and aortic wall (Law, unpublished observations).

Among the earliest attempts to measure ECS were those of Fenn[58], based upon the erroneous assumption that tissue chloride is entirely extracellular. Calculation of ECS on this basis does not result in grossly misleading values for whole body[202] or in tissues with low IC chloride (e.g. skeletal muscle[135], for review see Mannery[134]), but is clearly inapplicable to tissues in which there is a high concentration of IC chloride such as intestinal and vascular smooth muscle[31,99,102], cardiac muscle[151,163], renal cortex[204] and medulla[111]. It has been suggested that early techniques for chloride analysis gave erroneous values due to interference by other anions[40]. Sulphate has been widely used, as to a lesser extent has bromide (for review of the distribution of bromide in many rat and human tissues see Pierson et al.[158]). Iodide has proved useful in studies of cerebral fluid dynamics[2,50] but it extensively penetrates cells in some tissues (e.g. see Flear and Graber[60]). Like sulphate and bromide, thiosulphate[30,148,168] and thiocyanate[70,143], used in several earlier studies, have been found to occupy spaces greater than those available for markers of higher molecular weight, and their use has been largely superseded. Other anionic ECS markers include iodothalamate[90,93,170], poly-L-glutamate[119], ethanesulphonate[32,75,76] and cobalticyanide[74]. In recent years extensive use has been made of polyethylene glycols (PEG) which are obtainable with accurately graded molecular weights (e.g. 900, 4000). EDTA, labeled either with chromium[79,86,132,133,144,163,164] or cobalt[20,97,99], is regarded as a reliable ECS marker, and its use has been particularly recommended in muscular tissue due to its ability to penetrate tendon water[20]. Miscellaneous markers include cyanocobalamin[41,132,133] and creatinine[182]. Specialized applications have also been found for ^{22}- or ^{24}sodium[13,51,208], radio-iodinated serum albumin[19,33,45,47,132,133], lanthanum[127,137,190], radio-iodinated insulin[130,132,133], choline chloride and trimethyl-tris (hydroxymethyl)-ammonium chloride[81].

For a detailed treatment of the requisite physicochemical characteristics of ECS markers see Ling and Kromash[119].

Although for any tissue under given physiological conditions there must exist a unique anatomical space, it is amply documented (with a few exceptions, see for example Macchia et al.[126]) that the measured ECS diminishes as the molecular weight of the marker increases[20,24,37,38,53,75,115,145,163,173] – for reviews of earlier work see Elkington and Danowski[55], Kruhøffer[101] and Law[105]. The most plausible explanation of this would appear to be a) that markers of high molecular weight fail fully to penetrate the ECS or b) that those of lower molecular weight penetrate cells; but the choice of possibilities is not as simple as this, and molecular dimensions are only one of a number of factors affecting a marker's volume of distribution.

Factors likely to cause over-estimation of ECS

There is no firm evidence that any recognized ECS marker is rigidly excluded from cells, although larger markers may penetrate cells to a lesser extent and more slowly than smaller ones, and the latter retain their usefulness particularly in studies based on rapid single transit times. For example inulin, the largest marker in common use, slowly penetrates cells in the renal cortex[128], although for reasons associated with the method for its extraction from the tissue an over-estimation of ECS may not result from this[129,140]. Mention has already been made of the way in which ECS may be estimated even when cellular penetration is probable, by following the rapid and slow phases of marker uptake or efflux. Even in isolated tissues such as slices, in which markers do not have to cross a capillary wall, the fact that penetration of the ECS is not instantaneous remains a problem in classical equilibration experiments. ECS of slices may also be over-estimated due to entry of markers into damaged peripheral cells[4]. Some markers, notably sucrose[46,158] and raffinose[56], may be metabolized (e.g. by intestinal cells). The use of tritiated markers presents some special problems. It has been known for many years that the ^{3}H atoms exchange to a small but significant extent with H atoms in functional groups within tissues[165], which will clearly lead to over-estimation of ECS. In addition, several workers have drawn attention to the inhomogeneity of some commercially available preparations of ^{3}H-methoxy-inulin[56,124,145] – lower molecular weight fractions will penetrate cells to a greater extent than the pure compound. ^{35}S may similarly exchange with sulphur atoms in connective tissue mucopolysaccharides[116]. There is evidence that inulin may be accumulated by macrophages in the thyroid[35] and by hepatic phagocytes[194]: it may also be bound by connective tissue[197]. Bromide is sequestered by skin and gastric cells[158].

Factors likely to cause under-estimation of ECS

Under-estimation is normally due to the failure of a marker adequately to penetrate the ECS or do so at an unacceptably slow rate. Dense connective tissue partially resists marker penetration[148]. In some tissues inulin, and possibly other large marker molecules, are denied full EC distribution due to the exclusive effect of hyaluronic acid-rich mucopolysaccharides. The structure and function of mucopolysaccharide gels in connective tissue have been reviewed by Laurent[104] and their exclusive effects described by Ogston and Phelps[150]. Goresky[77] has examined the possible conse-

quences of the partitioning effects of mucopolysaccharides, in relation to molecular size, in the hepatic ISS. Significant increases in the inulin space have been reported following treatment with hyaluronidase in intestinal smooth muscle[75] and the crystalline lens[155], while recent evidence suggests that functionally important dilatations of the lateral cellular interspaces in rat renal collecting duct, previously observed histologically[67,199], may depend for their formation upon the action of a tissue-specific hyaluronidase[175].

Finally there are a number of miscellaneous factors which may affect the reliability of ECS measurements. Discrepant values for ECS in vivo and in vitro have been reported for skeletal muscle[106,115,121], cardiac muscle[121,151,163] and liver[87,154,197]. These presumably reflect differences in experimental method and do not necessarily invalidate the values obtained under the prevailing conditions. Anoxic deterioration can lead to histologically demonstrable increases in the inter-fibre spaces of skeletal[106] and cardiac muscle[25] and in brain ECS[141]. In some transporting epithelia differing spaces are found depending upon whether the marker is presented at the mucosal or serosal surface; a true estimate requires that it shall have access to both (see for example Nellans and Schultz[147]). In rat jejunum the discrepancy diminishes as the marker molecular weight increases[57]. The presence of the blood-brain barrier (BBB) imposes a similar constraint on determination of cerebral ECS[198]. In renal slices the size of the measured ECS depends upon the plane of section[94].

The penetration of highly charged markers, e.g. sulphate, may be impeded[126] or exaggerated[193] by the presence of fixed charges within tissues.

A variety of techniques independant of, or complementary to, the use of marker molecules exist for estimation of the ECS. Few seek to express ECS in precise quantitative terms; their usefulness consists mainly in their ability a) to detect rapid changes and b) to visualize small but significant changes in specialized areas of the ECS (e.g. lateral cellular interspaces). Autoradiography has been used to follow uptake of inulin[186] and PEG 900[157] by rabbit ileum. Although light or electron microscopy may be used as alternatives to chemical markers in determination of total tissue ECS (see for example Prosser et al.[166]), the principle use of these techniques lies in the examination of alterations in ECS which are too small to be detected by markers, e.g. in renal collecting duct[67,175,199] and gall bladder wall[185,200,201]. Histological examination of the ECS has been correlated with marker penetration in skeletal muscle[106], crystalline lens[155] and brain[141]. Artefactual distortion of the ECS is clearly a potential hazard in morphological studies, but freeze-substitution has been found satisfactory in cerebral tissue[82–84] and, with only minor variations, fixation in 1–2% osmium tetroxide at pH 7.2–7.8 has been successfully applied to gall bladder wall[127,191,201], brain[141] and renal medulla[175].

Alterations in the electrical resistivity and transepithelial electrical potential difference have provided valuable information regarding rapid and important changes in the dimensions of lateral cellular interspaces in renal tubules (for review see Boulpaep[15]), intestinal wall[127] (for review see Schultz et al.[180]) and gall bladder wall[127,185,200,201]. Fluctuations in the electrical impedence of cerebral tissue may be used to assess changes in the relative dimensions of the EC and IC compartments[82,85,89]. Comparable studies have been made using light-scattering technique[120] which has long been applicable to estimation of cell volumes in suspensions[8].

Reference has already been made to the application of EC markers to the study of capillary permeability and to the paired or multiple trace-dilution technique. Examples of these applications, which depend upon timed fractional venous collection of the markers following rapid injection of a single bolus, will be cited in section III. When the tracer-dilution technique is being employed to study the cellular uptake of metabolites by infused organs an advantage clearly derives from the use of EC markers and transported molecules of comparable size and diffusibility. For example, mannitol has been used in conjection with phenylalanine[29,131] and L-glucose[204], while D- and L-glucose provide a perfect combination[206]. For the theoretical background to the use of EC markers in the study of capillary permeability by tracer-dilution see Crone[42], Martín de Julián and Yudilevich[136] and Yudilevich[203] and for more wide-ranging discussion Crone[43].

III. *The extracellular space in individual tissues*

A) *Muscle*

1. *Skeletal muscle*

Although the ECS of mammalian skeletal muscle has been studied in small incubated segments[121], most investigations have relied on tissue analysis following perfusion in situ or in isolation, or determination of washout kinetics following equilibrium (for review of the latter see Garlick[69]). Soaking even relatively small muscles in vitro is unsatisfactory – e.g. there is a 2.5-fold increase in the sucrose space of soaked rat skeletal muscle by comparison with that in perfused muscle, probably due to anoxic deterioration[106,115]. Reported inulin spaces include 12.5% (unless stated otherwise, percentage spaces refer to ml · 100 g wet weight^{-1}) for rat muscle in vivo[1], 10% in rat gastrocnemius following isolated hind limb perfusion[115] and 13% in cat muscle[44], although a much higher value (32.5%) has been found in rat soleus[39]. As might be expected, rather larger spaces are obtained with smaller markers, e.g. in rat gastrocnemius 11% for raffinose[115], 12%[115] and 15%[126] for sucrose. Considerably

higher values are found in perfused cat gastrocnemius (sucrose 22%, mannitol 30%)[40]. Among ions, sulphate occupies spaces which do not greatly differ from those found with low molecular weight saccharides and (possibly due to charge effects) shows a significantly smaller ECS (10.5%) than sucrose in rat gastrocnemius[126]. A still lower figure (8%) for sucrose and EDTA has been obtained in rabbit quadriceps[163]. Bromide yields consistently larger spaces than sulphate in muscles of intact or nephrectomized rats and dogs[10] and thiosulphate also occupies a larger space than inulin in dog muscle[148].

There are relatively few studies in which ECS markers have been used as adjuncts to the investigation of physiological properties of skeletal muscle cells, but inulin has been used to assess the effects of insulin and membrane stabilizers on amino acid uptake by rat soleus[39] and the effects of insulin on cellular Na and K movement in the same tissue have been examined using sucrose[36]. For review of the application of EC markers to the study of capillary permeability in human skeletal muscle see Trap-Jensen and Lassen[192].

2. *Diaphragm*

Due to its structure, diaphragm can conveniently be examined with minimal tissue damage due to anoxia or cell destruction. Several studies have confirmed the existence of a quantitative relationship between the molecular weights of markers and their volumes of distribution. Thus Randle and Smith[168] obtained the following values in rat diaphragm - inulin 14%, raffinose 20%, sucrose 21%, thiosulphate 23%. Lower values (13%) have been found with EDTA and sucrose in the rabbit[163], while Ling and Kromash[119] suggest that in the rat the true ECS is as low as 9%. Diaphragm is one of the few tissues in which ethanesulphonate has been used: its volume of distribution varies between 18% and 24%[76]. Iodide extensively penetrates rat diaphragm[60].

As with skeletal muscle, there are few examples of EC markers in metabolic studies, but inulin has been used to measure accumulation of carnosine and β-alanine by rat hemidiaphragm[149].

3. *Heart*

There are marked differences in the ECS of different regions of the heart. In the rabbit sucrose spaces of 34% (atrium), 27% (right ventricle) and 22% (left ventricle) have been found in vivo[136]: a comparable figure has been obtained for right ventricle of the cat in vitro[177]. These regional differences presumably reflect mechanical function. Considerably higher but less stable values have been reported for rabbit heart on the basis of capillary permeability studies using inulin and sucrose[178] and in vitro[151]. Lower ventricular spaces (sucrose and sulphate, 20%[126]; sulphate, 19%[161]), and atrial space (sucrose, 25%[89]) have been found in rat heart. A mannitol space of $\sim 25\%$ has been measured in the whole organ[47]. Ventricular ECS, as assessed by EDTA, decreases during contraction[164]. The cardiac inulin space is unaffected by myocardial hypertrophy[138]. The sucrose space increases, even in non-ischaemic regions, following myocardial infarction[162].

In isolated bovine Purkinje fibres mannitol and sucrose spaces approaching 70% have been measured during investigations of Na efflux[16].

For theoretical consideration of the effects of lymphatic drainage on estimates of cardiac capillary permeability and ECS see Johnson[95].

The cellular mechanism of impaired glycollysis in ATP depletion has been studied by analysis of rat cardiac tissue following perfusion with EDTA[79]. Cell volume and K content have been derived from inulin spaces in perfused, contracting rat heart[152], and the kinetics of cardiac glucose uptake have been followed in the same species using mannitol as tracer[47]. In the latter study a vascular marker (serum albumin) was incorporated in order to allow assessment of the ISS ($\leqslant 5\%$). The characteristics of capillary permeability have recently been studied by measuring fractional venous collection of EDTA following rapid injection into dog heart[86] and of multiple tracers from isolated perfused rabbit heart[130].

4. *Vascular smooth muscle*

Vascular, and in particular arterial, walls possess a complex ECS[181]. EDTA indicates a value of $\sim 40\%$ in rat[97] and rabbit aorta[99], and a comparable value has been arrived at from anatomical considerations and the volumes of distribution of several markers in the carotid artery of the dog[193]. Values of about 50% have been reported for EDTA distribution in rabbit pulmonary artery and portal vein[99]. For further references see Burnstock[27] and Siegel et al.[181].

5. *Myometrium*

The ECS within uterine smooth muscle, like that of atrium and vascular walls, is relatively high. In the rabbit muscle inulin occupies a space of 47%, sorbitol and EDTA over 50%[20]. Slightly lower inulin spaces are found in the rat (37–41%) although the ethanesulphonate[32] and sorbitol[80] spaces approach 60%. Spaces do not vary between pregnant and non-pregnant individuals, and the volumes of marker distribution indicate constant IC concentrations of Na, K and Cl[32]. Theoretically, therefore, myometrial equilibrium potentials in this species should be unaffected by pregnancy. Closely similar sorbitol spaces (50–55%) have been recorded in both oestrogen- and progesterone-dominated rabbit uterus[97]. For further references see Burnstock[27,28].

For consideration of the intestinal smooth muscle see section III, B3.

B) *The gastrointestinal tract*

1. *Salivary glands*

EC markers have been extensively used by Yudilevich and his colleagues in studies on salivary gland physiology. Mannitol has been used as an EC reference tracer in order to follow amino acid uptake by cat submandibular glands by means of the paired tracer-dilution technique[29,131], and the permeability characteristics of the fenestrated salivary capillaries similarly investigated using a variety of EC and vascular markers[132,133].

2. *Gastric mucosa*

Fractional extraction of vascular and EC markers have been used in examination of gastric vascular permeability in the dog by techniques similar to those described in the preceeding subsection[3].

3. *Intestine*

Due to the diversity and importance of their transporting properties, the walls of the various intestinal segments have been widely studied. As in other tissues containing smooth muscle, the precise extent of the ECS is difficult to assess due to the relative impenetrability of the associated dense connective tissue (for discussion see Goodford[73]). For markers presented to both surfaces of rabbit ileal strips, inulin occupies 25% and mannitol 50% of the total tissue water[147], although the penetrative characteristics of inulin have been questioned on autoradiographic grounds[186]. In rat small intestine, mucosal and serosal compartments contribute approximately equally to a total ECS of ~ 16% - the serosal compartment equilibrates more slowly and at a rate dependent upon the rate of intestinal fluid transport[56]. The rate of transport of γ-amino*iso*butyric acid affects the apparent ECS in rat jejunum, probably due to opening of paracellular pathways[144]. Relatively higher spaces (as percentage tissue water) have been reported for the whole canine small intestine on the basis of mannitol and arabinose distribution[9]. For biopsy specimens of human duodenum an ECS of 15-18% has been measured using cyanocobalamin, and a 13% space with PEG4000[41]. Marked differences exist between the ECS of the various structural sub-components of intestinal wall - e.g. the inulin space (and rapidly equilibrating mannitol space) of canine small intestine in vivo are quoted as 27% (mucosa), 36% (sub-mucosa) and 26% (muscle)[79].

Inulin occupies 30-35% of guinea-pig *taenia coli*[20,75], the space being increased by hyaluronidase[75]. Sorbitol and sucrose spaces are ~ 40%[20,21,75]; that for EDTA is comparable or slightly higher[20,99]. For further references see Burnstock[27,28].

Extensive studies have been made on penetration of amino acids into intestinal tissue using EC markers to permit calculation of cellular accumulation, and stress has been laid upon the necessity for accurate delineation of the ECS if reliable cellular uptake kinetics are to be derived[156,157]. For example, the uptake of serine over a wide concentration range by rabbit ileum appears to occur at slightly but consistently different rates depending whether inulin or PEG900 is used as reference marker (although the differences were not significant in paired tracer studies)[156]. Trans- and paracellular routes for lysine uptake by rat jejunum have been studied using inulin and mannitol as EC markers[145], as has the uptake of glycyl-leucine by guinea-pig intestine[88]. PEG900 and inulin have been used, respectively, to follow methionine and *iso*leucine uptake[26] and β-alanine uptake[149] by isolated intestinal cells.

Electrolyte transport has been monitored in rabbit ileum, using inulin, in order to study its relationship to cell volume[177] and compartmentalisation of Na[184]. Mannitol has been employed as a marker in assessing intestinal Na and water transport following acute volume expansion in rats[91]. Investigations of ionic movement in smooth muscular tissues such as intestinal wall may be complicated by ion absorption onto extracellular solids, notably connective tissue[98].

4. *Liver*

Liver is an organ suitable for study by tissue slice technique, and as with brain (see section III, C1) the spaces within slices have been found to exceed those in the intact organ. Thus while inulin spaces of 25-30% have been recorded in slices[87,154] the hepatic ECS in nephrectomized rats appears to be only 10-12% immediately following i.v. injection of sucrose and inulin (although rising to ~ 25% after 8 h). Values of ~ 20% have been obtained shortly after i.p. injection of inulin[194] and during experiments on amino acid uptake by intact liver[1]. Mannitol spaces derived from tracer-dilution curves are far higher than would be expected from the distribution volumes of a smaller marker (^{22}Na)[22]. Determination of ECS in liver by means of marker equilibrium is complicated by drainage of the ISS by the biliary system[179].

Hepatic blood vessels are extremely leaky, and macromolecules readily penetrate at least part of the ISS. Thus while ^{51}Cr-tagged red cells suggest a 'true' vascular space of about 3.5%, serum albumin and polyvinypyrrolidone indicate an ISS of 6-7%[197]. For further discussion of the hepatic ISS see Goresky[77].

EC markers have been used in determining the effects of noradrenaline in K uptake by hepatocytes[87] and the dependence of their electrolyte content and ultrastructure upon various metabolic conditions[174,176]. The cellular fraction in suspensions of isolated hepatocytes has been calculated from measurements of the interstitial distribution of inulin[11,12].

5. *Pancreas*

L-glucose has been used as an EC marker in examination of the effects of insulin and pancreozymin on the

uptake of D-glucose and amino acids by exocrine cells in the isolated mouse pancreas[48].

6. *Gall bladder*

Interest in the ECS of gall bladder is centered on the question of transmural water and solute movement. Transport of Na has been monitored using inulin as EC marker[62] and the findings extrapolated to estimate cellular Cl content[52]. Methods based on microscopy, intercellular accumulation of La, and the electrical properties of bladder wall have all provided valuable information on trans- and paracellular transport processes under various metabolic conditions including osmotic stress[127,185,200,201] (for recent review see Whittembury et al.[196]).

C) *Nervous tissue*

1. *Brain*

The ECS of cerebral tissue may conveniently be examined by equilibrium of tissue slices, but possibly due to rapid post mortem changes the spaces measured in vitro greatly exceed those in the intact organ. The following comparison of distribution volumes has recently been made in rat brain - inulin, 22.4% (slices) vs 13.3% (in perfused brain); sucrose, 31.7% vs 16.6%; mannitol, 42.3% vs 19.5%[4]. Lund-Andersen[125] has described three phases for the washout of inulin from slices, of which the first may represent a true ECS of 29%, the later two being partly or wholly cellular. The initial phase of inulin washout indicates an ECS agreeing reasonably well with that shown morphologically[141]. Cold and anoxia depress the inulin and sulphate spaces in cortical slices - the decrement may represent a so-called 'second space' consisting of tubules ramifying within the cells and connected to the main ECS by micropores[37,38].

Measurement of ECS in intact brain is complicated by a) the unique properties of the BBB (see below) and b) draining of the interstitium by the CSF (the 'sink effect') (for review see Woodbury[198]). Thus, unless care is taken to achieve equilibrium, the ECS of perfused brain may be under-estimated if based on concentrations of marker in the plasma, or over-estimated on the basis of concentrations in the CSF. If both vascular and ventricular compartments are perfused to equilibrium the ISS is estimated at 15%[49,167]. Vascular perfusion with iodide (actively transported across the BBB) indicates a total ECS in rabbit cerebral cortex of between 22.5%[2] and 32%[50].

Transport of amino acids[208] and glucose[13] across the BBB of the dog have been estimated by tracer-dilution techniques using ^{22}Na as a vascular marker: the BBB is relatively impermeable to Na[51]. L-glucose may also be useful in such studies[207]. A technique has recently been developed for the rapid ($\leqslant 20$ sec) correlation of changes in regional blood flow and glucose uptake[72] (for review of glucose transport in brain see Lund-Andersen[125]). In the latter study, as in the use of short-term tracer-dilution technique, the 'sink effect' is nullified.

Passage of saccharide molecules across the BBB, like their interstitial distribution, is size dependent, and experiments making use of this have recently been carried out in order to characterize brain capillary permeability[5] and to trace the development of the BBB and cerebrospinal drainage of the ISS in foetal sheep[53]. Detailed discussion of the nature of the BBB lies outside the scope of this review, but it may be noted that serum albumin, normally vascularly confined, has proved useful (with PEG's) in elucidating the mode (convective vs diffusive) and anatomical basis for drainage of the ISS[19,45].

Finally, Hansen and Olsen's technique for assessing relative EC and IC volumes during cerebral ischaemia and cortical spreading depression[81] has been well supported by the results of the studies based on electrical impedence[85,89], electronmicroscopy[83,84] and light scattering[120].

2. *Superior cervical ganglia*

Inulin occupies a space of 32% in isolated rat superior cervical ganglia, sulphate 44%, while sucrose and mannitol yield intermediate spaces[19]. This is one of the few tissues in which tritiated compounds (mannitol and inulin) have been shown to define significantly large spaces than their carbon-labeled counterparts[23].

The efflux of γ-aminobutyric acid from ganglia has been followed using inulin as EC marker[17].

3. *Peripheral nerve*

The ECS of peripheral nerve is structurally complex, but is believed to be fully penetrated by sucrose and EDTA, which show a space of 36%[18].

D) *Crystalline lens*

Paterson[155] has correlated inulin distribution with electronmicroscopic observation of ECS in ox and rabbit lenses. The small inulin space ($\leqslant 5\%$) exceeds that visualized morphologically, and is increased by treatment with hyaluronidase. Higher values for distribution of mannitol and sucrose have been reported for rabbit, calf and rat lenses (10–12%[189]), while the sucrose space in sheep lens may be as high as 20%[103].

E) *Placenta*

Several tracer-dilution studies have been undertaken in order to evaluate placental accumulation of metabolites. Amino acid uptake into trophoblasts, from both foetal and maternal sides, has been examined in guinea-pigs using L-glucose as reference tracer[54,204,205], which allows correction for non-specific diffusion into adipocytes[123]. Similar experiments on uptake of D-glucose clearly indicate the stereospecificity of placental glucose transport[206] and of D- and

L-lactate uptake[118]. L-glucose has also been used to study placental uptake of prostaglandins from the foetal circulation[205].

F) *Lung*

The main interest in pulmonary ECS lies in the alteration of the relative dimensions of the EC and IC compartments which accompany oedema - these have been comprehensively reviewed by Straub[187]. As in the liver, serum albumin readily enters at least part of the ISS in isolated perfused lungs[33]. Reference has already been made to spaces in pulmonary vasculature (III, A4).

Fractional extraction of EC saccharides using double-tracer technique has been examined in order to determine pulmonary vascular permeability in dogs[42] and the uptake of fatty and amino acids. D-sugars and prostaglandins by rat lungs[188].

G) *Kidney*

The kidney is so structurally and functionally diverse that few attempts have been made to assess whole organ ECS. The theoretical implications of a washout kinetics using various vascular and EC markers have been discussed by Chinard[34].

Inulin has been widely used in the estimation of ECS in cortical slices (consisting mainly of proximal tubules); there is some disagreement regarding the influence of temperature and other metabolic conditions on the true size of the space, but most workers have reported values in the region of 25%[68,94,139,173,194,195] (for discussion and further references see Macknight[128]). Higher values are obtained if slices are cut sagittally[94]. Mannitol, dulcitol and sucrose penetrate renal cortex more extensively than inulin, but their volumes of distribution are reduced by phlorhizin (e.g. mannitol from 60% to 43%) which suggests that they penetrate cortical cells[173].

Inulin spaces in rat renal medulla increase from ~ 25% in the outer zone to over 40% in the papilla[108]. Higher values are obtained with sucrose[109]. In sheep kidney the entire medullary inulin space is 45%[68]. Thiocyanate appears to penetrate outer medullary cells[113] and there is a high cellular concentration of Cl[111].

Although inulin has been used to estimate the fluid trapped within the ECS of small fragments of separated cortical tubules[153,160], the functionally important changes in ECS occurring in the tubular cellular interspaces cannot conveniently be measured by conventional marker techniques. For review of micropuncture methods and measurements of transepithelial electrical phenomena in the elucidation of the functional role of the intracellular ECS see Boulpaep[15], Frömter[63] and Giebisch[71]. Precipitated La has been used to demonstrate the potentially permeable intercellular tight junctions which separate the luminal and peritubular components of the ECS[137,190] (see also Machen et al.[127]).

Recovery of inulin and amino acids from perfused kidney has been used to characterize the various modes of renal amino acid uptake[61] and creatinine has been employed as an EC marker in studies on brush border disaccharides activity[182]. Uptake of dipeptides by isolated rabbit brush border vesicles has been followed using inulin[66] and cellular Cl uptake by cells in the collecting duct followed by measuring inulin concentration changes in tubular fluid[100].

Amino acid transport by renal slices has been measured with sucrose as EC reference marker in cortex[172] and with inulin in both cortex and medulla[122]. Inulin has also been used to examine uptake of carnosine and β-alanine by rat renal cortex[149], penetration of Cl into guinea-pig cortical slices[144], accumulation of *para*-aminohippuric acid by rabbit cortical slices[139] and problems associated with anion exchange and cell volume control in slices of guinea-pig cortex[159].

Morgan[142] has used inulin in the detection of cellular volume changes in rat papillary cells exposed to high concentrations of NaCl, and there have been several related studies on cell volume control in slices of rat medulla under a variety of incubatory conditions[108-110, 112, 113]. This marker has also been used in the study of electrolyte movement in monolayers of cultured renal epithelial cells[183].

IV. *In conclusion*

In scientific research, unlike certain other human activities, techniques, once established, do not regress. There is no marker which has been shown beyond reasonable doubt to delineate ECS with complete accuracy under all conditions; and less direct methods, while invaluable for demonstration of small changes in specialized regions of the ECS, for the most part lack precise quantitative definition. But the work described in this review - and much more that I have been obliged reluctantly to omit - has provided a large corpus of valuable and reliable physiological information. There is no reason to suppose that in coming years this will grow both in magnitude and in precision.

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Linalool from the cotton stainer *Dysdercus superstitiosus* (F.) (Heteroptera: Pyrrhocoridae)

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Summary. The isoprenoid linalool, together with several straight carbon-chain aliphatic materials, has been found in the scent glands of the cotton stainer *Dysdercus superstitiosus* (F.)

Within the Hemiptera – Heteroptera (land and water bugs), nearly 30 species in 10 families are known to produce glandular secretions consisting predominantly of hydrocarbons and carbonyl compounds[2,3]. These secretions are believed to serve in defence since they are produced when the insects are disturbed or irritated and are often directed towards the source of irritation[4]. Of over 80 species of Heteroptera whose metathoracic gland secretions have been analyzed, only a few species have been reported to produce monoterpenes[5–7]. The few reports of isoprenoid constituents in the scent oils are thus of great interest and deserving of further attention. Here we would like to report our findings that the isoprenoid linalool together with several straight carbon-chain aliphatic materials (hexanal,

Composition of the scent volatiles from adult metathoracic gland and larva posterior abdominal gland (5th instar) of *D. superstitiosus*

Scent gland						Identification	Mass-spectrum (m/z)
Metathoracic gland			Abdominal gland				
Peak No.	R.T.	% R.A.	Peak No.	R.T.	% R.A.		
-	-	-	**1**	0.8	2	Hexanal	EI 100(M^+), 82, 72, 56, 44 Cl 101(M+1), 82
1	1.6	44.6	**2**	1.7	6.5	Hex-2-enal	EI 98(M^+), 83, 69, 55, 41 Cl, 99(M+1), 85
-	-	-	**3**	4.6	16	Tridecane	EI 184(M+1), 101, 99, 85, 71, 57, 43 CI, 183(M-1), 141, 112, 85
2	2.7	3.3	-	-	-	Hexanol	EI 84(M-18), 73, 69, 55, 43 CI, 101(M-1), 84
(a)	6.6	1.5	-	-	-	Linalool	EI 154(M^+), 136, 121, 93, 80, 71, 69, 55, 41
3							CI 153(M-1), 137
(b)	6.8	47.9	**4**	6.7	16.6	Oct-2-2nal	EI 126(M^+), 108, 83, 70, 55, 41 CI 127(M+1), 109, 85
4	9.6	3.5	**5**	9.5	38	4-oxo-hex-2-enal	EI 112(M^+), 83, 55, 41 CI 113(M+1), 85
5	40.2	0.7	**6**	39.9	20.4	4-oxo-oct-2-enal	EI 140(M^+), 125, 111, 98, 83, 85 CI 141(M+1), 95, 80

Abbreviation: R.T., retention time; % R.A., percent relative amount; EI, electron impact; CI, chemical ionization; m/z, mass/charge.