Our experiments demonstrate that the use of casein as a substrate, as opposed to serum albumin and haemoglobin, results in sharper borders between the zone of reaction and the area containing the undigested substrate. An advantage of the method of radial diffusion described above is that casein can be utilized as a substrate in studies on enzymes with any pH-optima. To illustrate this, if the agarose casein mixture is prepared at neutral or basic pH values and then the slides are immersed in buffer of low pH (e.g. 1 or 2 for pepsin studies), the casein precipitates in small aggregates which are homogenously distributed within the gel. It is not possible to get a homogenous dispersion of casein in the gel if it is mixed directly with agarose and buffer below pH 4.5, since the casein is precipitated and aggregated.

Electrophoresis followed by diffusion. This method has been developed in order to provide a rapid electrophoretical localization of protease inhibitors or activators in samples from body fluids and organ extracts.

Agarose electrophoresis (Veronal-HCl buffer, pH 8.6) is first performed on the solution already proved to contain inhibitors by e.g. radial diffusion. After electrophoretic separation, longitudinal slides are cut out from the gel into which the proteins have migrated (Figure 2A), and placed upon another agarose gel (Figure 2B). This gel contains 2.25% agarose and 0.1% substrate (casein). In the latter gel, a trough is punched and the enzyme solution is poured in. The incubation is performed at 37°C for 4–5 h. Molecules diffuse from the slices into the underlying gel. The enzyme in the longitudinal well diffuses into the gel and digests the substrate. However, in those positions where inhibiting substances have diffused from the applied slice there is no, or a reduced, digestion. These results are clearly observed after the undigested casein has been precipitated with acetic acid and stained with amido black (Figure 3).

Figure 3A shows one test where a trypsin inhibitor of porcine colostrum is localized to the γ-globulin region, and another test where the tryspin-inhibiting activity of the α-globulin region in porcine serum is shown. Figure 3B is a test of serum from a new-born piglet which ingested its first meal of colostrum 3 h earlier. Note the trypsin-inhibiting action of both serum and colostrum. Figure 3B also demonstrates that urine from a 14-hour-old suckling piglet exhibits trypsin-inhibiting activity in the γ-globulin zone. This may depend on the fact that a trypsin inhibitor actually has been absorbed from colostrum via the gut epithelium to the blood and then via the kidneys to the urine[5].

This is a good example of the applicability of this qualitative test in a situation when the measurement of the total trypsin-inhibiting activity would have given incomplete physiological information.

Zusammenfassung. Es werden zwei Methoden (Diffusion und Elektrophorese in Agarose-Kasein) für quantitative und qualitative Bestimmung von Inhibitoren und Aktivatoren verschiedener Proteasen beschrieben und die Trypsininhibitoren in Kolostrum, Serum und Urin von neugeborenen Schweinen besonders untersucht.

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A Double-Labeled Frozen Section Technique for Studying Distribution of H^3-Norepinephrine[1]

The arterial wall is stratified into tunicae intima, media and adventitia with differing diffusion characteristics and catecholamine uptake capacities. Catecholamines, either released from the sympathetic nerve terminals or applied exogenously, do not distribute uniformly throughout the arterial wall. Their distribution profiles are of physiological and pharmacological consequence[2].

A frozen section technique has been devised to study the transmural distribution of norepinephrine in the rabbit aortic strip[3]. By this technique, the kinetics of entry of tritiated norepinephrine (H^3–NE) into the arterial wall has been characterized. Briefly, the arterial strip was exposed to H^3–NE before it was frozen and sectioned into thin slices parallel to the intimal surface. The tritium content of each slice was assayed and plotted against the depth of the slice within the arterial wall to obtain a distribution profile[4]. As the catecholamines are subject to cellular uptake, differentiation betwen the cellular and extracellular components in the profile is often necessary. Rinsing the tissue with saline solution can largely remove the substance from the extracellular spaces, but this will most likely mobilize some of the cellular component as well. Further, rinsing is precluded in kinetic studies in which the tissue must be instantaneously frozen at the end of exposure to H^3–NE. This problem has been met by subtracting the H^3 material expected in the extracellular space from the total H^3 content to derive cellular component in each slice. The aortic extracellular space has been independently determined using the same frozen section technique but substituting C^{14}-inulin for H^3–NE[5]. The direct application of the average extracellular space (inulin space) thus obtained to studies of distribution of H^3–NE made on separate aortas, suffers from the considerable variations between aortas and between slices from the same aorta.

The present work is an attempt to circumvent this drawback. The aortic strip was doubly labeled with H^3–NE and C^{14}-inulin. Strips of the rabbit thoracic aorta were soaked in 0.32 μg/ml of C^{14}-inulin (inulin-carboxyl-C^{14}, 3.0 mc/g, New England Nuclear, Boston, Mass.) singly, or in combination with 0.5 μM H^3–NE (1-norepinephrine-7-H^3, Amersham/Searle, Arlington Heights, Ill.). After 55 min of soaking in a 10 ml tissue bath, for an additional 5 min the strips were superfused with the same medium at a rate of 3 ml/min. At the end of superfusion, the tissues were immediately frozen, sectioned at 24 μm and

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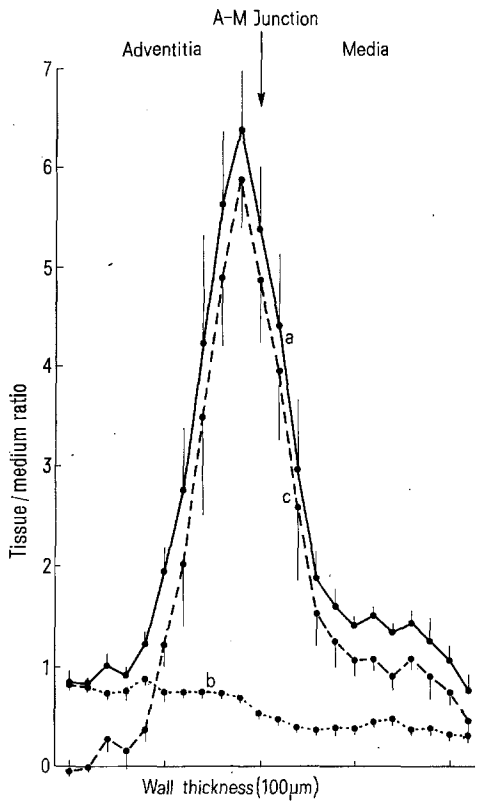
processed as earlier[4]. The H^3 and C^{14} activities in each slice were simultaneously counted by a Unilux II Liquid Scintillation System (Nuclear-Chicago Corp.) using window settings appropriate for doubly labeled counting.

In 5 experiments in which C^{14}-inulin alone was applied, the inulin space profile observed earlier[5] was confirmed. Namely, the space was significantly lower in the media than in adventitia. The results were essentially the same as those shown in curve b in the Figure. Disregarding the transitional zone (the slice containing the adventitio-medial junction and 2 slices on either side), the media and adventitia gave inulin spaces of 0.37 ± 0.02 and 0.78 ± 0.04 ml/g (mean $\pm$ S.E.M.), respectively, in terms of the tissue/medium ratio (i.e. dpm per gram tissue to dpm per ml medium). The space in these aortic strips as a whole was 0.57 ± 0.02 ml/g.



Distribution profiles of H^3 (curve a), C^{14} (curve b) and the difference between H^3 and C^{14} (curve c) in the wall of rabbit aorta. Aortic strips were exposed to a mixture of H^3-NE and C^{14}-insulin for 1 h (see text). Each point represents the mean value of 5 determinations and each vertical bar the standard error of the mean. The slices of all strips corresponding to the adventitio-medial junction were grouped and other slices accordingly aligned for calculations.

In 5 other experiments, H^3–NE and C^{14}-inulin were applied simultaneously. The H^3 distribution profile formed a well-defined peak (Figure, curve a) with a maximum situated slightly to the adventitial side of the adventitio-medial junction where the adrenergic nerve terminals are known to be concentrated. The tissue/medium ratio at the peak ranged from 6 to 8. These agreed with previous results obtained by using H^3–NE alone[6]. Therefore, there was no evidence that the presence of C^{14}-inulin altered the distribution or uptake of H^3–NE under the experimental conditions. Similarly, since the C^{14} profile in the presence of H^3–NE (curve b) was essentially the same as in its absence, H^3–NE did not seem to affect the distribution of C^{14}-inulin. When the inulin space is subtracted from the H^3 tissue/medium ratio in each slice, this gives the profile of H^3–NE presumably bound to cellular and intracellular sites (curve c).

The H^3 and C^{14} tissue/medium ratios in all slices were significantly different ($p < 0.05$) except the 4 slices in the outermost adventitia. This suggests no uptake of H^3–NE in the adventitial regions which lack the nerve terminals. On the other hand, all medial slices, even those remote from the adventitio-medial junction, showed significant uptake. Excluding the 2 innermost slices which may contain the intimal endothelium and subendothelial elastic lamella, and taking the next 4 innermost slices, the net H^3 tissue/medium ratio was 0.98 ± 0.07. This presumably represents the uptake of H^3–NE mainly by the smooth muscle cells. It indicates that these cells 'clear' H^3–NE from a volume of the medium approximately equivalent to their own volume.

This technique can obviously be extended to other pairs than C^{14}-inulin and H^3–NE, so far as they do not interfere with their respective diffusion and uptake, and to tissues other than aorta. It seems to be particularly useful in kinetic studies of movement of substances within a tissue and where cellular uptake is expected.

Zusammenfassung. Beschreibung einer verbesserten Methodik zur Differenzierung der intra- von der extrazellulären Verteilung des exogenen Noradrenalins in der Aortenwand des Kaninchens.

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CONGRESSUS

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