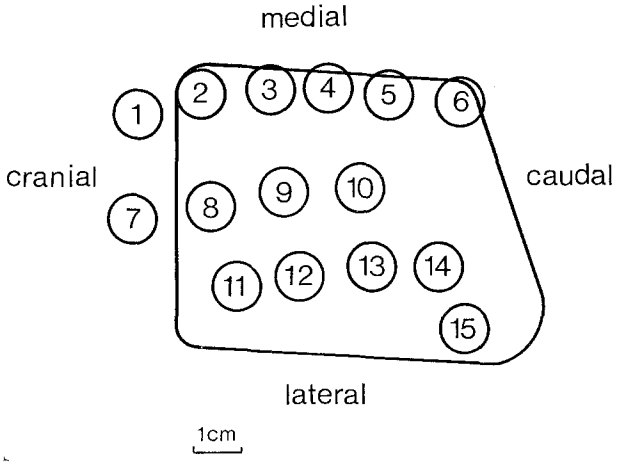
messer von rund 0,3 mm verfertigten, in den Glasstopfen lediglich eingehängt und nicht eingeschmolzen. Das Absorptionsmedium (6 ml Methanol) wird erst nach abgelaufener Verbrennung durch ein Septum im Schraubverschluss des Stopfens eingespritzt. Nach Schütteln des Kolbens werden nach etwa 15 min 5 ml der Absorptionsflüssigkeit in ein Zählgläschen zu 10 ml Szintillations-Lösung (7 g Butyl-PBD/l Toluol purum) pipettiert. Diese Modifikation erlaubt ein rascheres und ungefährlicheres Arbeiten. Die Proben wurden in einem Beckman-LS-150-Flüssigkeits-Szintillations-Spektrometer mit externer Standardisierung zur Ausbeutebestimmung gezählt.

Probenentnahmenstellen



Resultate und Diskussion. Die Messwerte der verbrannten Gewebsstückchen nach Abzug des Blindwertes sind in der Tabelle zusammengestellt. Die Doppelbestimmungen wurden nicht gleichzeitig durchgeführt. Die Werte von inaktiven Vergleichsproben lagen zwischen 70 und 90 cpm. Die angeführten Standardabweichungen widerspiegeln lediglich die Unsicherheiten, die sich aus dem radioaktiven Zerfall, der Instabilität des Zählgerätes und dem wechselnden Einfluss der kosmischen Strahlung ergaben. Nicht erfasst wurden Kontaminationen mit Fremdaktivitäten, Chemiluminiszenzerscheinungen oder Fehler bei der Probenvorbereitung.

Auf das 40 cm² grosse Applikationsfeld waren 200 µCi ^{3}H-Flumethason aufgetropft worden. In einem ausgestanzten Hautstück von 1 cm Durchmesser waren also ursprünglich durchschnittlich ca. 4 µCi ^{3}H-Flumethason vorhanden. Dies sind bei einem durchschnittlichen Trockengewicht der Stücke von 90 mg ca. 97000 dpm/mg Epidermis. Die mittlere gefundene Aktivität der Epidermisproben betrug nur noch 5000 dpm/mg. 92000 dpm, also ca. 95%, hatten die Epidermis innert 2 h demnach bereits passiert. Da die tieferen Schichten praktisch keine Aktivitäten enthielten, wurde somit die radioaktive Substanz sogleich durch den Blutstrom in den Unterhautgefässen abtransportiert. Es ist also offenbar nicht möglich, durch dermale Applikation von Flumethason im Gewebe unterhalb des Coriums wesentliche Wirkstoffkonzentrationen zu erreichen. Wenn Flumethason als sehr schlecht wasserlösliche, apolare und lipophile Substanz vom Blut quantitativ abtransportiert wird, ist dasselbe Verhalten auch von der Grosszahl der übrigen pharmakologisch aktiven Substanzen mit zumeist polarer Natur zu erwarten. Die klinischen Erfolge bei der perkutanen Behandlung von Gelenks- und Muskelaffektionen des Pferdes beruhen deshalb nicht auf einer Anreicherung der Wirkstoffe unterhalb der Applikationsstelle, sondern viel eher auf einer reflektorischen Änderung der Zirkulationsverhältnisse im geschädigten Gewebe, ausgelöst durch die Reizung der Rezeptoren im behandelten Hautstück (kutiviszerale Reflexe in den entsprechenden Headschen Zonen).

Summary. Two h after the application of 200 µCi ^{3}H-Flumethasone (0.73 mg) to the shaved skin of a horse, only 5% of the substance could be found on or in the treated epidermis. The deeper tissues contained practically no radioactivity. Therefore, dermal application does not appear to be an effective means of treating these tissues.

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Retention of Matrix Density in Adrenergic Vesicles After Extensive Norepinephrine Depletion

Two types of vesicles which contain norepinephrine (NE) are found in adrenergic nerves. Large dense core vesicles (LDV) are always present both in the axons and terminals, while small dense core vesicles (SDV) generally predominate in the terminals. However, the distribution of the two vesicle types varies greatly not only in different species but also at various loci in the same species. It is not known to what degree each type contributes in the release of NE and other substances upon nerve stimulation. The bovine splenic nerve contains a relatively high proportion of LDV in the terminal varicosities, where they are estimated to occur in a 50–50 distribution with SDV[1], which makes it tempting to infer a direct role for these vesicles in transmitter release.

There have been many attempts to correlate the vesicle NE content and/or other matrix components with the electron density after various treatments (see reviews[2–4]). Initial glutaraldehyde fixation followed by osmium tetroxide preserves essentially all NE in isolated LDV from splenic nerve throughout fixation and dehydration procedures prior to embedment for electron microscopy, as demonstrated by radioactive tracer retention studies[5]. The study of several different fixation and staining

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methods led to the conclusion that the staining reaction in the LDV matrix was due to complexing substances, rather than to transmitter per se.

Materials and methods. Bovine splenic nerves were excised, partially dissected and chilled within 10 to 12 min post mortem. LDV were released by mild Ultra Turrax homogenization and purified by a sequence of fractional, sucrose-D_2O density gradient and a final fractional centrifugation as described earlier in detail[6]. Aliquots of the purest vesicle fraction, $FIII_{M15}$, were suspended in a medium containing 0.1 *M* sucrose and 0.1 *M* potassium phosphate buffer at pH 7.4 and maintained at 0–4 °C for the duration of the experiment. These were cold controls. Experimental LDV were incubated in the above medium also containing 5 m*M* $MgCl_2$, 5 m*M* $tris_4$ATP and 0.5–1.0 μg ^{3}H-l-NE/ml for 45 min at 30 °C. Pargyline HCl at a final concentration of 50 μ*M* was added to all media in order to inhibit the small remaining monoamineoxidase activity.

After 45 min, equal volumes of LDV suspensions and 4% purified glutaraldehyde in 0.05 *M* sucrose buffered with 0.2 *M* potassium phosphate at pH 7.4 were mixed and centrifuged at 140,000 g_{max}-40 min. The resulting pellets were treated with 2% osmium tetroxide in the same buffer for 15–30 min, washed, stained en bloc in 1–2% aqueous unbuffered uranylacetate, dehydrated rapidly and embedded[7].

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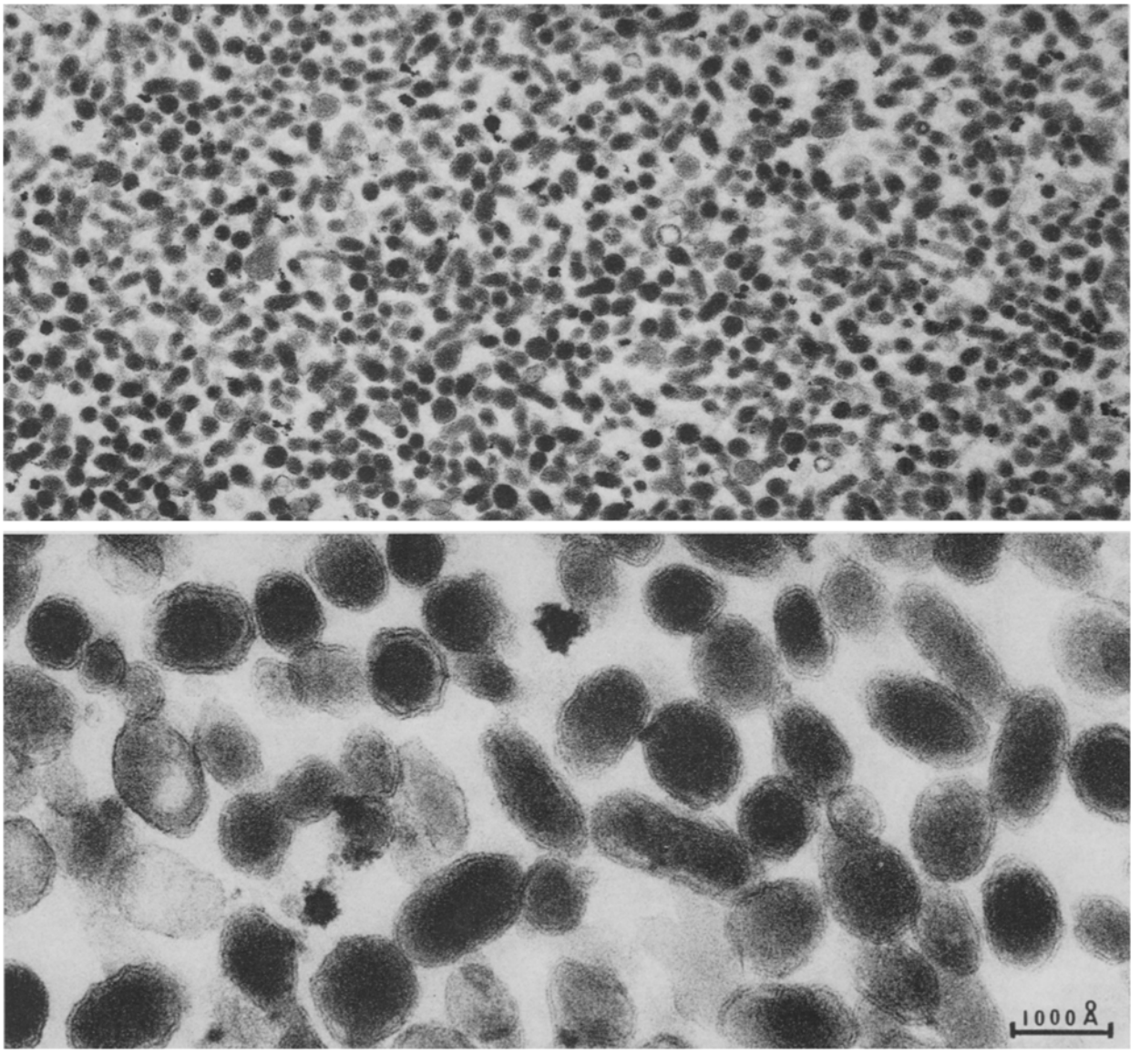

Fig. 1 and 2. Purified LDV incubated for 45 min at 30 °C in the presence of 5 m*M* Mg^{++}-ATP and 0.5–1.0 μg l-NE/ml. The equivalent of 1 m*M* ATP was added at each 10 min interval during incubation to counterbalance hydrolysis. The vesicles in the micrographs contained 2.5 μg NE/mg protein after 45 min incubation. Thus, they had undergone 77% depletion from the cold control value of 10.8 μg NE/mg protein. Figure 1 shows a portion of the vesicle pellet close to the surface with nearly all vesicles filled with electron dense material. Dense amorphous granules are present among the vesicles. The extravesicular granules increase during all incubation procedures, but especially when conditions compromise vesicle membrane integrity. ×30,000. Figure 2 shows the LDV at higher magnification and clearly reveals the limiting unit membrane of each vesicle. ×140,000.

NE was analyzed spectrophotofluorometrically using a trihydroxyindole method after alumina column purification of 0.4 *M* perchloric acid extracts of unfixed pellets or supernatants. After isotope equilibrium, ^{3}H-l-NE retention in fixed pellets was compared with duplicate unfixed controls by dissolving both pellets in Nuclear Chicago Solubilizer and counting extracts in a Beckman LS-250 β-scintillator with automatic quench compensation[5].

Results and discussion. The routine LDV preparation consists of 80–90% pure vesicles with essentially all contaminating particles restricted to the bottom 5–10% layer of the fixed pellet. The LDV contain an average of $\sim$ 10 µg NE/mg protein (range 8.7 – 12.3; $N = 9$), when nerves are obtained and chilled after a minimal 10 to 12 min post mortem delay. Electron microscopic observation reveals that nearly all cold control LDV contain a finely granular, dense matrix material. After increasing periods of post mortem delay (eg. 20–30 min or longer) or after incubation at 30 °C for 45 min in the absence of Mg^{++} and ATP, the LDV become progressively more translucent[7]. Under these conditions it appears that the vesicles lose NE as well as other components which are involved in the staining reaction. Therefore, the matrix density semi-quantitatively parallels the NE content.

However, when LDV with an initially high NE content are incubated for 45 min at 30 °C in the presence of Mg^{++} and ATP, they can lose up to 70–80% of their original NE without any obvious decrease in the density of the matrix staining reaction (Figure 1) and without destruction of vesicle membranes (Figure 2). In experiments with either Mg^{++} or ATP omitted, it is found that each enhances or maintains the matrix density, with ATP having the major effect.

It is still not understood how adrenergic vesicles maintain their NE stores, and a number of factors appear to be involved in the binding of transmitter[8–10]. The proteins dopamine β-hydroxylase (DβH) and chromogranin A are released along with NE upon nerve stimulation[11,12]. These proteins and possibly other chromogranins or precursors are present in LDV[13,14] along with ATP[8], cholesterol, phosphatidylethanolamine and lecithin[13], and the cations, Cu^{++} and probably Mg^{++} and Ca^{++}.

LDV fixed in glutaraldehyde followed by osmium tetroxide or potassium dichromate become sufficiently dense to be discerned easily under the electron microscope without further staining by heavy metals[5]. However, treatment with uranylacetate will considerably increase contrast. This is particularly evident when the pellets are stained en bloc for a period of 15–30 min at room temperature before dehydration. This enhances the contrast of vesicle membranes and the density of the matrix material superior to staining sections on the grid. It suggests that uranylacetate treatment before dehydration prevents alcohol extraction of some material originally present in the LDV. Others have shown that brain tissue treated with uranylacetate lose less phospholipid and only $^1/_4$ as much protein as untreated tissue during subsequent dehydration[15]. Uranylacetate also reacts with both saturated and unsaturated lecithin and tends to solidify lecithin monolayers in vitro[16]. These data indicate that a portion of the dense material retained in the LDV and stained by uranylacetate is likely to be protein and/or phospholipid.

It can be speculated that some of the present staining reaction is also due to ATP. The high affinity of uranyl ions for nuclei acids, presumably the nucleotide phosphate groups, is well recognized and the hypothesized ATP complex within the LDV matrix could provide similar uranyl binding sites. This possibility is in keeping with the earlier observation[17] that vesicles partially depleted of NE after a 20–30 min post mortem delay are relatively translucent but increase in density after incubation in the presence of Mg^{++} and ATP, even though the NE content is augmented very little if at all.

There is also some evidence that the staining reaction in part depends on a macromolecular complex containing high DβH activity[18]. This enzyme was recently reported to be a Cu^{++}-glycoprotein[19]. DβH is the major protein released during LDV incubation and more of this enzyme is released into the supernatant after 15–30 min incubation of purified vesicles in the absence of ATP[20]. Concurrent with the loss of DβH and of densely staining material from the vesicles, there is an increase in extravesicular granules which also stain densely with uranylacetate.

It was recently hypothesizied[10] that LDV are packaged in the perikaryon with proteins and some NE. They are transported along the axon towards the terminals where they first become enriched with NE and then release their soluble contents by exocytosis. The enrichment of vesicle NE content during axonal transport has been confirmed at the purified LDV level[21]. The speculated 'all or none' process of exocytosis for LDV is relatively inefficient in typical long post ganglionic adrenergic fibres, if functional DβH and other protein constituents have to be replenished by synthesis in the perikaryon. The present finding in vitro that a major release of transmitter can occur from LDV in the presence of Mg^{++} and ATP with little or no loss of other substances responsible for the vesicle matrix staining reaction may be physiologically pertinent. It suggests the possibility that an ATP-regulated neuronal control mechanism may operate in vivo, whereby nerve activity can result in NE release with minimal concomitant loss of substances essential for transmitter synthesis and complex formation. The direct correlation between NE content and the density of the matrix staining reaction in vesicles isolated from nerves after increasing periods of post mortem delay, on the other hand, could indicate that neuroplasmic ATP levels soon become insufficient to maintain the matrix components.

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Zusammenfassung. Es wird gezeigt, dass die elektronenopaken Matrizen der adrenergen Vesikel nach Inkubation mit ATP und Magnesium gut erhalten sind, und zwar trotzdem der Gehalt an Katecholamin bis zu 75% verloren ging. Die Elektronendichte kann mit Uranylazetatfärbung gesteigert werden.

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11 March 1974.

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A Study on Amino Acids in the Gastric Mucosa During Differentiation and their Significance. II

As a means of isolating the epithelial cells of the mucosa, the 33% alcohol immersion method is well known. FUJIE[1] has succeeded in extracting some contents in the surface epithelial cells of the rat gastric mucosa by this method, and has proved by the amino acids analyzer that dozens of amino acids and several kinds of related compounds exist in the alcohol. MABUCHI[2] proved experimentally that lysine, histidine, arginine and tyrosine have the effect of promoting production of secretory granules in the gastric chief cells of rats.

The author[3] reported in a previous paper that the amount of amino acids in the differentiating gastric mucosa is very small; in the developing mucosa it increases regularly day by day, but the increase of the restricted amino acids – lysine, histidine, arginine, tyrosine and isoleucine – is delayed more than that of others, and the beginning of this increase coincides with the first appearance of rich secretory granules in the gastric chief cells.

To investigate further the effect of lysine, histidine, arginine, tyrosine and isoleucine, the author examined their influence on the embryos when pregnant rats were given with these amino acids.

Table I. Dose and kind of amino acids and related compounds injected into pregnant rats

Substance	Group A	Group B
Tryptophan		0.2
Lysine	1.6	
Histidine	0.4	
Arginine	0.7	
Cysteic acid		1.5
Taurine		23.7
Aspartic acid		2.9
Threonine		2.8
Serine		4.9
Glutamic acid		16.9
Proline		2.0
Glycine		9.8
Alanine		7.8
Valine		1.4
Cystine		0.7
Methionine		0.6
Isoleucine	0.7	
Leucine		1.7
Tyrosine	0.8	
Phenylalanine		0.8

Dose injected is $\times 10$ μM of the value ascertained from 1 g-stomach of adult rats fasting for 24 h.

Materials and methods. Wistar rats weighting 200–250 g were used for the experiment. The estrus female rat was kept in a wire cage with a male for a night. The next day was calculated as the 1st day of pregnancy. Pregnant rats were classified into A, B and C groups. They were injected with amino acids, as shown in Table I, throughout pregnancy. C-group rats were kept as controls.

Neonates of A, B or C group were classified in the same way into A, B or C groups, and materials were taken from the neonates on the 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23 postnatal day. To take materials, the neonates were separated from their mothers for more than 6 h, while the infant rats were not given food for more than 12 h. After weighing, the thorax and the abdomen were opened under deep ether narcosis, and Luna's liquid was injected into the artery from the left heart ventricle for the vital fixation. Small pieces taken from the stomach (glandular portion, if distinguishable) were put into Zenker's or Kolster's fixative for the post fixation. Paraffin sections of 7 μm were cut from the material fixed by the former fixative and stained by hematoxylin-eosin for histological observation; 4 μm sections, on the other hand, were cut from the stomach fixed by the latter fixative and stained by Heidenhain's iron hematoxylin for cytological observation of the chief cells.

Observations. The body weight of the neonates and the infant rats is shown in Table II. All the neonates, regardless of grouping, are covered with short white hair on the 7th–9th postnatal day, their eyes open on the 13th–15th postnatal day period.

1. *Histological observation.* The gastric mucosa on the 1st postnatal day is quite similar to that of the control rats, i.e. the mucosa forms very small plicae and acidophil cells can be distinguished in the epithelium. On the 3rd postnatal day, cell proliferation and pit formation at the basal part of the plicae can be observed. This may be interpreted as a tendency towards formation of the tubular glands. At this stage, this tendency seems more pronounced in rats of A group than in those of B and C groups. In the 5th postnatal day, the mucosal development is the same in the 3 groups. Thus, it may be presumed that the development from the 3rd to 5th postnatal day in B or C group rats may have already begun during the 1st–3rd postnatal day period in A group. The mucosa of A, B and C group rats on the 7, 9, 11, 13, 15, 17, 19, 21 and 23

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