coupled with the lack of normal electron staining, made it difficult to locate zonulae. However one could sometimes make out the typical narrowing of the gap between the cells, etc. The deposits were continuous through these regions. In addition, it is known that the zonulae (including z. occludentes) are almost universal in the larger lymphatics, especially the thoracic duct[3]. The fact that the deposits often ran continuously from the lumens to the adventitia showed that the zonulae must be permeable to ions. (This permeability of the z. occludentes and adhaerentes has been established in other sites, including the brain and retina[7-9].)

As might have been expected, the endothelial vesicles also often contained precipitates. No doubt, since they periodically open to the exterior, they must take in ions and transport them just as they do the large molecules[7,9-13]. However, as mentioned earlier, it is most unlikely that they contribute significantly to the passage of the small molecules.

It is evident, therefore, that the endothelial intercellular junctions of the large lymphatics, although closed to large molecules, are freely permeable to small ones. There seems little doubt that these correspond to the 'pores' of PAPPENHEIMER[14]. There also seems little doubt that it is via these junctions that the small molecules escape from the lymphatic system. The high hydrostatic pressures generated by the contractions of the walls of the collecting lymphatics[15,16], to which may be added the effects of gravity[1,2], must force considerable amounts of the small molecules out of the vessels. No doubt they rapidly pass out of the tissues into neighbouring blood vessels. If the lymphatics are carrying off large volumes of fluid from active muscles or oedematous regions, it is evident that the local high tissue pressures will prevent most of the fluid from leaving the vessels until they pass through a normal, inactive region. Then the escape will commence. Hence the lymphatic system can be considered to carry large molecules, almost without loss, to the various lymphatico-venous anastemoses; small molecules are carried out of active or oedematous regions, then they tend to pass into the blood system via the lymphatic walls and the tissues[17].

Résumé. Les petites molécules s'échappent des grands vaisseaux lymphatiques grâce aux jonctions endothéliales intercellulaires, qui ne laissent pas passer les grandes molécules.

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Effect of Calcitonin on Glycosaminoglycan Synthesis by Embryo Calf Bone Cells in vitro

Calcitonin lowers serum calcium by inhibiting bone resorption[1-3]. Crude calcitonin preparations have been shown to stimulate the incorporation of ^{14}C-glucose into glycosaminoglycan (GAG) synthesis by embryo calf bone cells in culture[4]. Increases ranged from 25–40% with hormone preparations of activities 0.2–2.5 MRC U/mg. The present work consists of further experiments to demonstrate this effect, and to study the effect of high specific-activity calcitonin on GAG synthesis.

The methods of establishing the cultures and measuring ^{14}C-GAG production by cells have been described previously[4]. In essence, cells were grown in medium 199 containing 20% human serum (HS), 10% foetal calf serum (FCS) and ^{14}C-glucose in polystyrene flasks half of which contained calcitonin. After 18–22 h culture the ^{14}C-GAG's were precipitated, washed, dissolved and counted. Calcitonin prepared by phenolic extraction and TCA precipitation[5] (TCA-CT, 0.45 MRC U/mg) consistently caused an increased ^{14}C-GAG synthesis (Table I). However no effect was obtained with preparations of high specific activity (Lilly-CT, 50 U/mg; Armour-CT, 58 U/mg; CIBA-CT, 55 U/mg), even at concentrations of 1 U/ml. One explanation of this failure was that the more purified preparations lost biological activity under the conditions of culture. This was investigated by assaying Armour-CT after incubation at 37 °C for 24 h in medium 199 with and without serum. TCA-CT in medium 199 and serum was assayed at the same time. Armour-CT lost all activity while the crude preparation retained some hypocalcaemic activity (Table II). The results in Table III indicate that the rate of inactivation of hormone is very rapid, since Lilly-CT had no detectable biological activity after 90 min.

Further experiments were aimed at growing the embryo calf bone cells in media containing considerably less serum, with a view to avoiding hormone inactivation.

Table I. Effect of TCA-CT (0.45 U/mg) on ^{14}C-glucose incorporation into GAG synthesis

Cell density (cells/ml)	^{14}C-glucose (μc/ml)	Calcitonin (mU/ml)	^{14}C-GAG synthesis (cpm) control	treated	p
120,000	2.0	22.5	251 ± 17 (3)	382 ± 38 (3)	< 0.05
70,000	8.0	40	2295 ± 88 (5)	2662 ± 90 (5)	< 0.02

Replicate cultures were grown in medium 199 with 20% HS, 10% FCS for 20 h; mean ± S.E.M. of cpm above background; No. of flasks in parenthesis.

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Table II. Effect of incubation on hypocalcaemic activity of Armour-CT and TCA-calcitonin

Preparation	Potency	Medium	Calcitonin concentration (mU/ml)	Serum calcium levels of assay rats (mg %) controls	low dose	high dose
Armour-CT	58 U/mg	20% HS 10% FCS in medium 199	279	9.99 ± 0.14 (6)	9.75 ± 0.15 (6)	9.66 ± 0.16 (6)
Armour-CT	58 U/mg	medium 199	279	9.34 ± 0.20 (5)	8.50 ± 0.15 (5)	7.43 ± 0.19 (4)
TCA-calcitonin	0.45 U/mg	20% HS 10% FCS in medium 199	279	9.45 ± 0.16 (6)	9.40 ± 0.14 (6)	8.50 ± 0.22 (6)

After incubation at 37 °C for 24 h, 0.4 ml samples of medium containing hormone were injected i.v. into fasting 90 g rats (high dose); 0.4 ml of 1:4 dilution (low dose); 0.4 ml of medium alone (control); blood sampled at 50 min; serum calcium estimated by fluorometry[8].

Table III. Rate of loss of calcitonin activity in culture

Time (min)	Group	Serum calcium (mg %)	p
0	Controls	9.70 ± 0.06 (6)	
	Lilly-CT (low dose)	9.20 ± 0.13 (6)	< 0.001
	Lilly-CT (high dose)	8.30 ± 0.06 (6)	< 0.001
90	Lilly-CT (low dose)	9.80 ± 0.09 (6)	n.s.
	Lilly-CT (high dose)	9.80 ± 0.29 (6)	n.s.

Lilly-CT (50 U/mg) was incubated at 37° with cells and medium at dose levels corresponding to 56 and 14 mU/0.4 ml, respectively; 0.4 ml samples taken at 0 and 90 min after addition of hormone and injected immediately i.v. into fasting rats; blood taken for serum calcium after 50 min; control rats injected with 0.4 ml culture medium only; means ± S.E.M.; No. of rats in parenthesis.

Table IV. Effect of CIBA-CT (58 U/mg) on ^{14}C-glucose incorporation into GAG synthesis

Cell density (cells/ml)	^{14}C-glucose (μc/ml)	^{14}C-GAG synthesis (cpm/flask)		p
70,000	0.8	controls	214 ± 10.2 (6)	
		CSL-CT (200 mU/ml)	484 ± 43.4 (6)	< 0.001
		CIBA-CT (1 U/ml)	401 ± 15.8 (6)	< 0.001
40,000	1.0	controls	156 ± 14.5 (6)	
		CIBA-CT (600 mU/ml)	268 ± 17.7 (6)	< 0.001

Replicate cultures were grown in medium 199 with 2% FCS, 1% HS for 18 h; mean ± S.E.M. of cpm above background; No. of flasks in parenthesis.

With as little as 1% FCS in medium 199, the subcultured cells appeared to adhere to the surfaces of the flasks in a satisfactory manner, but showed little secretory activity. With 2% FCS and 1% HS however, the cells incorporated appreciable amounts of ^{14}C-glucose into GAG synthesis. The effects of CIBA-CT and of crude calcitonin prepared by ethanol extraction[6] (CSL-CT, 1.5 U/mg) were studied. The results of these experiments (Table IV) showed a striking effect of both preparations of calcitonin on ^{14}C-glucose incorporation into GAG synthesis. The effect was much greater than that which had been seen in any of our early experiments with crude hormone, suggesting that biological activity was better preserved with less serum in the medium.

In the present state of knowledge of mechanisms of calcification it is not possible to reach any firm decision regarding the role of the GAG's in the process. The subject has recently been reviewed by BOWNESS[7], who concluded that the GAG's provide a greater calcium-binding capacity than any other known tissue constituent in the region of endochondral calcification, and that variations of the level of GAG's in calcifying regions can provide a sensitive control of the rate or the extent of the reaction between calcium and phosphate. The in vitro finding that calcitonin increases the synthesis of GAG's by cells closely related to bone provokes the suggestion that calcitonin may act by causing the synthesis and release of GAG's within the zones of bone resorption. The polyanions would bind calcium locally, giving the appearance of inhibition of bone resorption. Such a mechanism is consistent with the effects of calcitonin which have been observed in organ culture[1] and in short-term isotope experiments in vivo[2,3]. It ascribes to the hormone a stimulatory function which explains its apparent inhibitory effect on bone resorption[9,10].

Zusammenfassung. Es wurde gefunden, dass rohe Calcitoninpräparate die Inkorporation von ^{14}C-Glucose in die Glycosaminoglycan-(GAG)-Synthese fördern. Unter denselben Zellkulturbedingungen wurde Calcitonin mit stark spezifischer Aktivität rasch inaktiviert und zeigte keine stimulierende Wirkung; wurde aber der Serumgehalt im Milieu stark eingeschränkt, so verursachte das Calcitonin (50 Einheiten/mg) eine hundertprozentige Erhöhung der ^{14}C-GAG-Synthese.

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