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TOMUS LIX

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Physiologia—Pathophysiologia

INFLUENCE OF AUGMENTATION OF EXCRETORY RENAL MASS ON RENAL FUNCTION AFTER α-RECEPTOR BLOCKADE

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The effect of renal function of an augmentation of the excretory renal mass was investigated in 10 dogs without drug treatment and in 10 animals with α-receptor blockade.

In the untreated group, augmentation of excretory renal mass by transplantation into the neck of one pair of kidneys isolated from another animal caused the following changes in the kidneys *in situ*: marked elevation in C_{PAH}, slight decrease in C_{inulin}, slight diminution of urine excretion and a pronounced fall in sodium excretion. The amount of urine and sodium excreted by the four kidneys was identical with that previously excreted by the two kidneys *in situ*.

In animals with α-receptor blockade, augmentation of the excretory renal mass had the following consequences in the *in situ* kidneys, C_{PAH}, and C_{inulin} remained unchanged while urine and sodium excretion decreased to the same extent as in the untreated control group. The amount of urine and of sodium excreted by the four kidneys was the same as that excreted by the kidneys *in situ*, prior to transplantation of isolated kidneys, *i.e.* before the augmentation of excretory renal mass.

It seems that the decrease in sodium excretion of the kidneys *in situ* was not due to the haemodynamic changes evoked by the load on the circulation; it was rather consequence of some quick, presumably humoral, regulation.

The diminution of sodium excretion in the kidneys *in situ* after augmentation of the excretory renal mass has been ascribed to an increased utilization by the four kidneys of the natriuretic factor(s), *i.e.* to a diminution in the plasma level of the natriuretic hormone.

Unilateral nephrectomy causes an about twofold increase in the urine and salt excretion of the persisting kidneys without any change in salt and water intake. Thus, even one kidney is capable of maintaining the salt and water equilibrium of the organism. The polyuria observed in the persisting kidney is presumably due to an alteration in the function of the nephrons; the mechanism of the phenomenon, however, is not clear.

It has been shown that renal excretion is regulated by several factors beside alterations in glomerular filtration rate (GFR) and aldosterone secretion. Recently, increasing attention has been paid to the role of the so-called natriuretic factor(s). Independently of the fact whether or not the hypothetic natriuretic hormone is renal [23, 26, 31] or extrarenal [13] in origin, the diminution or augmentation of the excretory renal mass will certainly influence

the rate of hormone utilization and, thereby, the effect of the hormone on the kidney.

In previous experiments [18] we have studied the influence of the augmentation of secretory renal mass on the function of the kidneys. After transplantation of isolated kidneys, *i.e.* when the excretory renal mass had been augmented, a definite decrease was seen in the urine and sodium excretion of the kidneys *in situ*. These findings have been explained by an increased utilization of the natriuretic factor. Transplantation of the isolated kidneys (alteration of circulatory resistance) was, however, accompanied by a decrease of the PAH and inulin clearance of the kidneys *in situ*. Thus, it could not been clarified to what extent was the diminution in diuresis and natriuresis to be ascribed to the altered renal haemodynamics and to the increased utilization of the natriuretic factor. In the present experiments the effect on renal function of the augmentation of excretory renal mass has been studied after α-receptor blockade, an intervention excluding or minimizing the effect of transplantation on renal haemodynamics.

Materials and Methods

The experiments were performed on mongrel dogs of both sexes under intravenous pentobarbital 25 mg/kg anaesthesia. Body weight of the donor animals ranged between 10 and 12 kg, and that of the perfusors between 20 and 30 kg.

The animals were deprived of food for 24 hours prior to the experiment.

The isolated kidneys were transplanted on the neck of the perfusor dogs. These animals were divided in two groups. One group served as control and received no drug treatment either prior to, or during, the experiment. The second group consisted of animals which received a daily subcutaneous dose of 0.75 mg/kg of reserpine for three days before the experiments, in order to deplete the noradrenaline stores. Then, at the beginning of the experiments, 15 mg/kg of phentolamine was given in one injection intravenously; during the experiment proper the drug was administered at a rate of 10 μg/kg/min in intravenous infusion.

After anaesthesia, the perfusor animals received Ringer solution of body temperature, in an amount corresponding to 1% of their body weight. Thereafter, the femoral artery and vein were prepared and cannulated on both sides. The bladder was exposed through inferior median laparatomy, and polyethylene catheters were introduced into the urethers supravesicularly.

The left carotid artery and the right jugular vein were prepared from a median cervical incision and cannulated with glass cannulae.

Paraamino hippuric acid (PAH) and inulin were dissolved in 40 ml Ringer solution of body temperature. As a priming dose this solution was injected intravenously in such an amount as to produce 2 mg/dl and 30 mg plasma concentrations, respectively. The maintaining dose was infused intravenously at a rate of 0.25 mg/kg/min. After the infusion we waited 60 min for the equilibrium state to set in; this was followed by urine collection for 3×10 min control periods. At the middle of the urine collection periods, arterial blood samples were taken for determination of plasma PAH and inulin concentrations. Blood samples were centrifuged immediately; after removing the plasma we suspended the red corpuscles in physiological saline and re-infused them intravenously. Arterial blood pressure in one of the femoral arteries was continuously measured by a mercury manometer and recorded kymographically.

Blood clotting was prevented by the intravenous injection of 0.1 mg/kg of heparin.

After the control period the kidneys of another dog isolated according to 5 were connected with the circulation. The donor animals were anaesthetized and eviscerated, the suprarenal veins ligated and the kidneys removed together with a piece of the aorta and inferior caval vein. The isolated kidneys were then connected to the circulation of the perfusor animal

through the common carotid and the aorta stump as well as the stump of the inferior caval vein and the external jugular vein.

Thin polyethylene cannulae were introduced in the ureters. The duration of renal anoxia during transplantation did not exceed two minutes.

After transplantation of the isolated kidneys we waited ten minutes for the new equilibrium to set in, then the function of the *in situ* and isolated kidneys was investigated during two consecutive 10 min periods.

Thereafter, 25 ml/kg Ringer solution was infused into the perfusor animal over 60 minutes, during which the changes in the parameters of the *in situ* and the isolated kidneys were followed for three more consecutive 20 min periods.

Renal blood flow of the isolated kidneys (outflow from the inferior caval vein) was measured at the middle of each period, using a graduated cylinder and stop watch.

PAH concentration in the urine and plasma was determined according to SMITH et al. [29]; inulin according to LITTLE [22], sodium and potassium by flame photometry, and osmotic concentration with *Fiske*'s osmometer. The haematocrit value was determined by means of *Hawskley*'s microhaematocrit, and plasma protein concentration with the biuret method.

Clearances (C_{PAH}, C_{inulin}) were calculated according to the usual formula. The values given refer to 100 g of kidney weight, except the vascular resistance of isolated kidneys, which was calculated for kg of renal mass ($R_{kidney/kg}$). For statistical analysis either the one tailed or two-tailed *t* test was used.

Results

Renal parameters were studied in 10 non-treated (control) dogs and in 10 animals with α-receptor blockade (in the following), these animals will be referred to as blocked dogs. The first three periods in the Tables show the parameters measured in the *in situ* kidneys during the three 10 min urine collection periods prior to transplantation of the isolated kidneys. In Period 4 and Period 5 the function of the isolated and of the *in situ* kidneys, respectively, was studied after transplantation of the isolated kidneys. Thereafter, EC hypervolaemia was augmented according to the procedure described in Methods, and the changes in the parameters of the kidneys were followed for three 20 min periods (Periods 6—7—8). The Figures show the mean values and SEM., the full line the control and the broken one the blocked animals.

Figure 1 and Fig. 2 show the parameters of the kidneys *in situ*.

As seen in Fig. 1, mean arterial blood pressure in the control animals was 132 ± 6 mm Hg at the beginning with no essential change either during, or at the end of the experiments (137 ± 4 mm Hg). In the blocked animals the initial blood pressure amounted to 103 ± 4 mm Hg; it did hardly change and remained significantly lower than in the control group throughout the experiment ($p < 0.001$).

Initial C_{PAH} was 235 ± 12 ml/min in the control and 231 ± 19 ml/min in the blocked animals. During the three control periods there was no significant difference between the two groups. After transplantation of the isolated kidneys C_{PAH} decreased from 251 ± 16 ml/min to 186 ± 15 ml/min in the control dogs ($p < 0.001$) and remained at this low level throughout the experiment. In the blocked animals C_{PAH} did not change during the experiment.

Initial C_{inulin} was 75.5 ± 6.3 ml/min in the control and 79 ± 6.9 ml/min in the blocked group. After transplantation of the isolated kidneys the value

n the *in situ* kidneys of the control animals was 72.0 ± 6.3 ml/min and decreased to 64.9 ± 1.9 ml/min ($p < 0.05$). Then, with progressive augmentation of the extracellular hypervolaemia it began to increase and reached 84.8 ± 7.6 ml/min by the end of the experiment. In the blocked animals, C_{inulin} of the *in situ* kidneys did not change after transplantation of the isolated kidneys; then, upon the effect of hydration, it increased to 92.0 ± 5.6 ml/kg by the end of the experiment.

Figure 2 shows the changes in urine and sodium excretion as well as those in the osmotic concentration of the urine of the kidneys *in situ*.

Initial urine excretion was 1.21 ± 0.21 ml/min in the control group and 1.52 ± 0.27 ml/min in the blocked group. The difference was not significant statistically ($p > 0.60$). During the third control period the values were quasi-identical, being 1.73 ± 0.37 ml/min and 1.79 ± 0.38 ml/min, respectively. Urine excretion slightly decreased in both groups after transplantation of the isolated kidneys, from 1.73 ± 0.37 ml/min to 1.27 ± 0.30 ml/min in the control group ($p < 0.05$) and from 1.79 ± 0.38 ml/min to 1.22 ± 0.24 ml/min in the

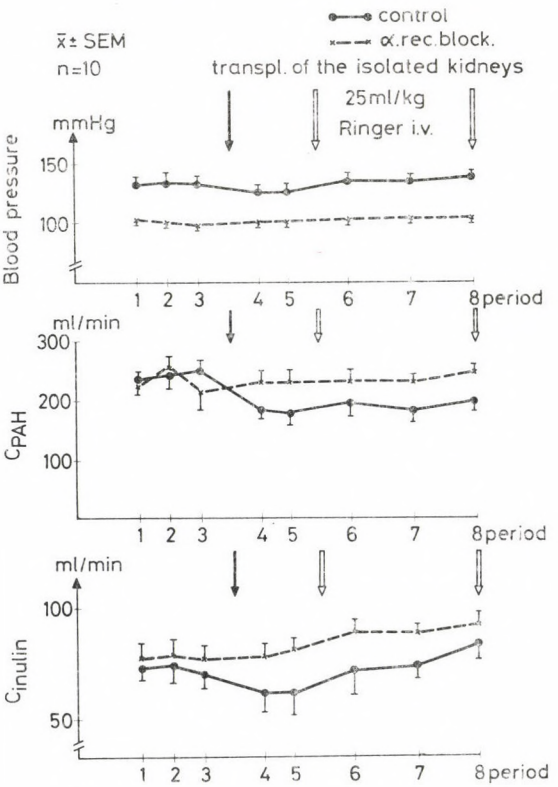

Fig. 1. Parameters of the kidneys *in situ* in the course of the experiments. Full line = control (untreated); broken line = animals with α-receptor blockade

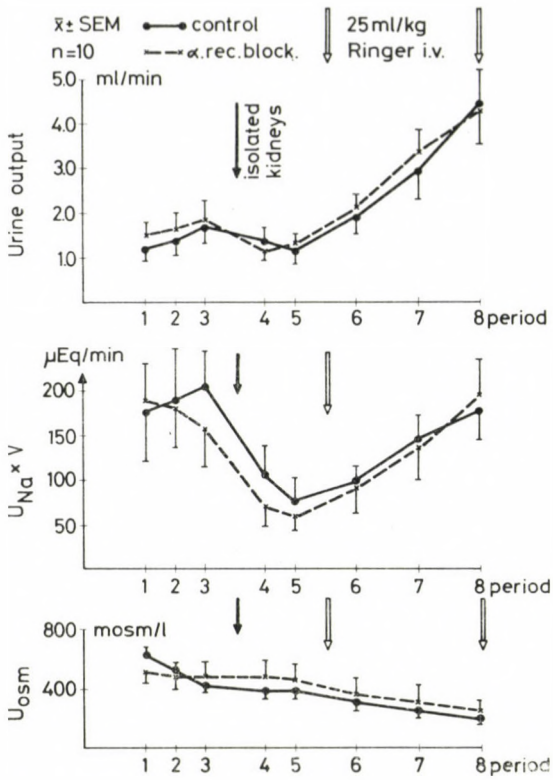

Fig. 2. Urine and sodium excretion and osmotic pressure of the kidneys *in situ*. For explanation see Fig. 1

blocked group. Hydration caused an augmentation in both groups; by the end of the experiment the values were 4.44 ± 0.85 ml/min in the control and 4.44 ± 0.88 ml/min in the blocked animals.

Sodium excretion was practically the same in both groups. After transplantation of the isolated kidneys it fell from 206 ± 61 μEq/min to 104 ± 31 and, later, to 78 ± 24 μEq/min in the control animals ($p < 0.01$), and from 158 ± 42 μEq/min to 70 ± 18 and 60 ± 16 μEq/min, respectively, in the blocked dogs ($p < 0.01$).

Hydration caused sodium excretion to increase to 180 ± 30 μEq/min in the control group and to 195 ± 62 μEq/min in the blocked group. At the beginning, total osmolarity of the urine amounted to 632 ± 36 mosm/l in control animals and to 525 ± 78 mosm/l in the blocked group. The difference was not significant statistically.

Transplantation of the isolated kidneys failed to cause any change in total osmotic activity of the urine obtained from the kidneys *in situ*. Then,

following hydration, it decreased below the osmotic activity of the plasma in both experimental groups. At the end of the experiment, osmotic concentration of the urine was 213±30 mosm/l in the control animals and 238±95 mosm/l in the blocked dogs.

Figures 3 and 4 show the changes in the parameters of the isolated kidneys. Period 4 and Period 5 correspond to the mean values obtained during the two 10 min periods following transplantation of the isolated kidneys, while Periods 6 to 8 show the mean values during the three 20 min hydration periods.

As seen in Fig. 4, renal blood flow in the isolated kidneys perfused from the control group was 493±35 ml/min at the beginning and 514±38 ml/min during the second perfusion period. Upon the effect of hydration it exhibited a slight initial decrease to 415±45 ml/min (NS) and reached the value of 468±46 by the end of hydration. In animals perfused from blocked donors the initial RBF in the isolated kidneys was 537±39 ml/min, with no further change throughout the experiment.

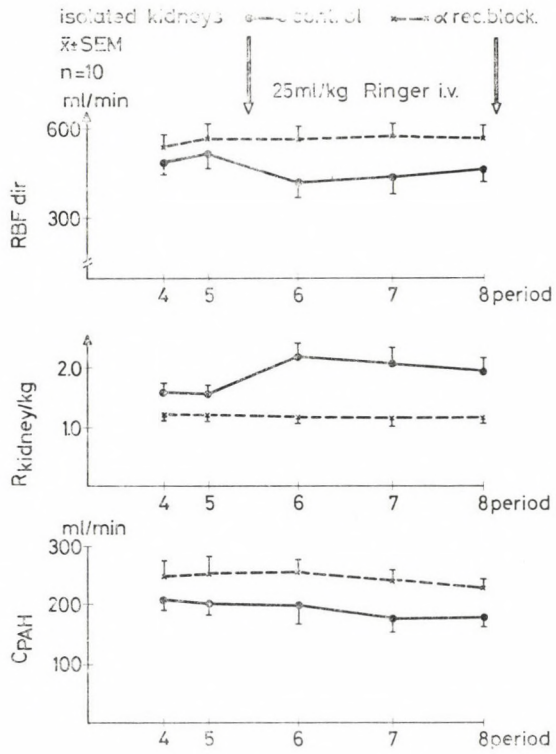

Fig. 3. Blood flow, renal resistance and PAH clearance of isolated kidneys. Period 4 and Period 5 show the values obtained during the two urine collection periods after transplantation of isolated kidneys. Periods 6—7—8 demonstrate the results obtained after augmentation of the EC hypervolaemia. Full line = control; broken line = parameters of isolated kidneys perfused from α-receptor blocked animals

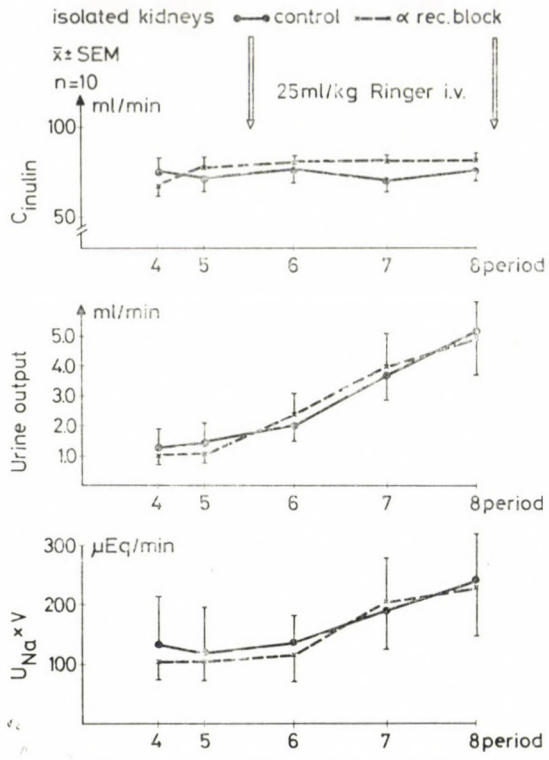

Fig. 4. Inulin clearance, urine and sodium excretion of isolated kidneys. For explanation see Fig. 3

At the beginning of perfusion, vascular resistance was 1.59 ± 0.13 in the isolated kidneys perfused from control animals and 1.23 ± 0.05 in the kidneys perfused from the blocked dogs ($p < 0.02$).

Vascular resistance of the kidneys perfused from control animals slightly augmented at the beginning of hydration; the value was 2.16 ± 0.25 and it remained significantly higher ($p < 0.01$) than that of the isolated kidneys perfused from blocked dogs.

Initial C_{PAH} was 210 ± 14 ml/min in the isolated kidneys perfused from control animals and 246 ± 30 ml/min in those perfused from the blocked group. The values remained practically unchanged in both groups, the difference between them being at the limits of mathematical significance ($p = 0.05$).

Figure 4 shows that the C_{inulin} values of the two groups were in good agreement, and that they did not undergo any significant alteration during the experiment.

At the beginning of the perfusion, urine excretion was 1.27 ± 0.64 ml/min in the group perfused from control dogs and 1.05 ± 0.25 ml/min in that per-

Table I

Plasma parameters

		Control			Transplantation of isolated kidneys		Hydration		
		1	2	3	4	5	6	7	8
Plasma Na^+	C	143±1.58	144±2.53	143±3.16	142±2.84	141±1.26	142±2.53	140±1.26	141±1.58
maeq/l	B	147±3.16	146±3.17	148±3.16	148±3.10	148±3.48	148±3.48	152±3.16	151±3.26
Plasma K^+	C	4.06±0.33	3.88±0.25	3.95±0.32	3.48±0.19	3.42±0.23	3.61±0.16	3.70±0.19	3.76±0.16
maeq/l	B	3.92±0.37	3.57±0.31	3.61±0.26	3.15±0.29	3.27±0.30	3.31±0.28	3.39±0.22	3.57±0.25
Plasma osm.	C	308±6.0	306±5.4	304±5.5	307±4.1	311±5.7	309±3.5	310±3.5	311±3.8
mosm/l	B	309±5.6	310±5.8	311±4.2	320±6.8	315±5.2	321±4.2	324±4.6	324±4.8
Ht%	C	39±1.31	39±1.46	41±1.38	44±1.19	44±1.38	39±1.18	37±1.63	36±1.67
	B	41±2.02	42±2.02	42±2.08	42±1.04	43±2.27	43±1.61	40±1.61	39±2.47
Plasma protein level	C	5.60±0.35	5.72±0.36	5.99±0.27	5.73±0.36	5.55±0.34	5.11±0.31	5.00±0.26	4.71±0.28
g/dl	B	4.90±0.14	4.94±0.14	4.90±0.16	4.82±0.14	4.70±0.13	4.36±0.12	4.15±0.15	4.10±0.17

$\bar{x}$ ±S.E.M. C: Control, B: animals with α-receptor blockade, n = 10

fused from α-receptor blocked animals; the difference was not significat statistically ($p > 0.60$). As a rule, hydration augmented urine excretion in both groups; at the end of the experiment the values were 5.15 ± 0.13 ml/min in the control and 5.04 ± 1.24 ml/min in the α-receptor blocked animals.

At the beginning of the perfusion, sodium excretion was 133 ± 84 μEq/min in the control group and 108 ± 47 μEq/min in animals perfused from α-receptor blocked donors (NS). Hydration caused an increased sodium excretion in both groups: the corresponding values were 240 ± 79 μEq/min and 230 μEq/min, respectively, at the beginning of the experiment.

The changes of plasma parameters are shown in Table I. Disregarding the diminution of the haematocrit value, hydration failed to cause any significant alteration in plasma parameters, neither did the prehydration parameters significantly differ in the two groups.

Discussion

The amount of sodium and water excreted by the kidneys is given by the difference between the amount filtered in the glomeruli and the amount re-absorbed in the tubuli. Due to the glomerulo-tubular equilibrium, changes in GFR influence only slightly the excreted amount of sodium and water, much greater changes are caused by occasional alterations in tubular reabsorption. Tubular sodium transport can be influenced by 1) changes in the plasma concentration of mineralocorticoids; 2) changes in the sodium concentration of the plasma; 3) changes in the Starling forces in the peritubular capillaries; 4) activity of renal afferent nerves; 5) presence and amount of occasional natriuretic factor(s).

The short duration of our experiments makes it unlikely that an alteration of the plasma aldosterone level should be responsible for the changes in sodium excretion observed during perfusion of the isolated kidneys. Hypernatraemia decreases while hyponatraemia increases the sodium re-absorption in the tubules. Since plasma sodium concentration did not change in our experiments, neither can this factor be made responsible for the diminished sodium excretion of the kidneys *in situ* following transplantation of the isolated kidneys.

Proximal water and sodium absorption may considerably be influenced by intrarenal physical factors. It is profoundly altered by changes of the colloid osmotic pressure in the peritubular capillaries; these changes are consequences of alterations in plasma protein concentration [11, 14, 18, 19, 20, 24]. In the present investigations, however, transplantation of the isolated kidneys was not followed by any change in plasma protein level; thus, neither this factor can be responsible for the altered sodium excretion.

It is true that, in the control series of experiment, the transplantation of the isolated kidneys was followed by a slight, non-significant decrease in the arterial blood pressure of the perfusor animal, and that C_{PAH} and C_{inulin} diminished significantly in the kidneys *in situ*. These factors could influence urine and sodium excretion partly by changing the hydrostatic pressure in the peritubular capillaries and partly by altering the tubular load. The alterations of urine and sodium excretion were, however, identical in both the control and the α-blocked animals, and no post-transplantation change occurred in the above parameters of the latter group after transplantation of the isolated kidneys. It is therefore assumed that the above-mentioned factors were not responsible for the diminished sodium excretion.

The phenomenon of denervation polyuria and natriuresis has often been investigated. The majority of the authors ascribes the phenomenon to a diminished water and sodium excretion in the proximal tubuli [1, 2, 3, 4, 10, 28, 30]. The increase of sodium absorption in the proximal tubuli after stimulation of the renal nerves has recently been attributed to an α-dependent mechanism [10, 28]. If this were the case, sodium excretion should have been at unchanged C_{PAH} and GFR augmented in our α-receptor blocked animals. This was, however, not the case, so the finding has probably to be ascribed to the decreased arterial blood pressure of these animals. Decreased arterial blood pressure can namely lead to a diminution of hydrostatic pressure in the peritubular capillaries. As a consequence, re-absorption by the switch-off of the adrenergic mechanisms.

Transplantation of the isolated kidney was followed by a significant and quantitatively identical decrease in sodium excretion of the kidneys *in situ* in both the control and the α-receptor blocked groups. Therefore, we do not ascribe any significance to α-mimetic mechanisms in the phenomenon. Nevertheless, such mechanisms do influence re-absorption in the proximal tubuli.

KNOX et al. [16] observed increased sodium excretion under the effect of an augmentation of blood volume, without any change in either RBF or GFR. This observation is indicative of the existence of such a circulating humoral substance that is capable of augmenting sodium excretion without altering the renal haemodynamics. The investigations of KALOYANIDES and AZER [15] and of LICHARDUS and NIZET [21] led to a similar conclusion. RECTOR [25] assumed the presence of a certain "factor" in the plasma of dogs and rats loaded with physiological saline; this factor would decrease sodium re-absorption in the proximal tubuli. The investigations of GONICK and SALDANHA [13] indicate that natriuretic factor(s) may be formed not only in the kidneys but in other organs as well. According to GODON [12], the natriuretic factor can modify sodium transport both in the kidney and extracellularly.

Though the chemical structure and the origin of the natriuretic factor(s) are not yet elucidated, there is no doubt about the existence of one or more substances which can modify sodium excretion without altering renal haemodynamics; it is also certain that neither of them is identical with aldosterone [6, 7, 8, 9].

The extent of inhibition of sodium transport in the nephron depends on the amount of the natriuretic factor.

When a pair of isolated kidneys was connected with the circulation of the perfusor animals, C_{PAH} decreased in the kidneys of the control animals, together with a slight diminution of the GFR. At the same time, there was some decrease in urine excretion and a fall to 1/2 or 1/3 of the control value in sodium excretion. Practically identical changes were found in the urine and sodium excretion of the α-receptor blocked animals, but without haemodynamic alterations and no change in GFR. This finding indicates that the decreased sodium excretion of the kidneys *in situ* after transplantation of the isolated ones is not due to haemodynamic changes. At the beginning of the perfusion of the isolated kidneys, their sodium excretion fell to half or one-third of the original value; at the same time, the amount of sodium excreted by the four kidneys (*in situ* + isolated) was the same as the amount excreted by two kidneys before perfusion. This shows that sodium excretion is under a precise and flexible regulation that adapts itself to new conditions within a few minutes. Similar results were obtained from a comparison of the present findings with those obtained in previous experiments (Tost et al. [32]). In the latter study no transplantation of isolated kidneys was performed and the response of the kidneys *in situ* investigated during an EC hypervolaemia of similar extent (25 ml/kg of Ringer solution intravenously over 60 min). In those experiments sodium excretion was 405 ± 77 μEq/min at the beginning of hydration; in the present study, the corresponding value was 180 ± 79 μEq/min for the kidneys *in situ* and 240 ± 79 μEq/min for the isolated ones. This means that the four kidneys together excreted the same amount of sodium as did two kidneys in previous experiments performed under otherwise identical conditions.

The results of our experiments can easily be explained in such a way that the natriuretic factor is utilized to a greater extent by four than by two kidneys. The natriuretic factor can namely be assumed to undergo inactivation while it affects the permeability in the nephron. Thus, when four kidneys are connected to the circulation, the natriuretic factor will be destroyed more rapidly, and its plasma concentration decreases; these events will then be manifest in an antinatriuretic action.

Comparison of the parameters of isolated kidneys in the two experimental groups revealed that renal blood flow and C_{PAH} were higher, while renal resistance was lower in the control than in the α-receptor blocked animals.

At the same time, the inulin clearance as well as the urine and sodium excretion of the isolated kidneys were identical in both groups. Neither urine nor sodium excretion was elevated in the α-receptor blocked animals, in spite of the high RBF. This can be explained by the significantly lower blood pressure in this group of dogs.

Summing up, it seems that the diminution of sodium excretion in the kidneys *in situ* after augmentation of the functioning renal mass is very likely due to an increased utilization by the four kidneys of the natriuretic factor(s).

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EFFECT OF HISTAMINE AND BRADYKININ ON LYMPH AND TISSUE FLUID COMPOSITION IN THE RABBIT HINDLIMB: EVIDENCE FOR TWO COMPARTMENTS IN TISSUE FLUID*

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In the rabbit hindleg lymph and tissue fluid were collected before and during close intraarterial infusions of histamine (2.5 and 10 μg/min) or bradykinin (0.4 μg/min). Protein concentration was higher in the tissue fluid than in the lymph. During the 2h of the experiment in the control animals protein concentration increased in the lymph and decreased in the tissue fluid. Lymph flow decreased in the controls and it was not significantly influenced by histamine but was in the majority of animals (14 of 20) grossly augmented by bradykinin. Lymphatic protein and LDH fluxes were increased both by bradykinin and histamine. In animals where the vasoactive agents failed to alter lymph flow LDH concentration in the lymph increased significantly without comparable changes in the tissue fluid. On the other hand, in experiments where lymph flow was increased lymphatic LDH activity decreased but the activity in tissue fluid remained unchanged. It is concluded that the interstitial fluid is composed of two compartments. Only the perivascular compartment of tissue fluid is drained directly by the lymph vessels and lymph is essentially a recent microvascular filtrate. The second compartment of the interstitial fluid is rinsing the cells and connective tissue fibres, it is parallelly coupled and in exchange with the perivascular compartment.

Recently, it has been shown that there are marked differences between the composition of subcutaneous or muscle tissue fluid and that of the lymph draining from the same region. In dogs and rabbits protein concentration was higher in tissue fluid than in lymph [12] and in rabbits activity of the cellular enzymes was also higher in tissue fluid than in simultaneously collected lymph samples [12, 14]. The observations suggested the presence of two compartments in tissue fluid.

The above hypothesis has been corroborated by observations made during ischaemic and burn injury where it was found that the activity of the enzymes escaping from the injured cells rise to much higher levels in tissue fluid than in the regional lymph [12, 15]. The procedures led to widespread tissue injury compounded with a lesion of the microvascular wall. In the present investigations the effects of histamine and bradykinin, i.e. substances

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increasing microvascular permeability and formed or released during local tissue reactions, were studied in rabbits. It was reported previously that in the dog and cat limb both histamine and bradykinin increased lymph flow and lymphatic protein concentration, eventually leading to oedema formation [3, 4, 5, 6, 10].

Materials and Methods

Rabbits of both sexes with a body weight of 3.5 to 4.0 kg were anaesthetized with an intravenous injection of 30 mg/kg pentobarbital. A plastic cannula was introduced in one of the afferent lymph vessels of the popliteal node. The carotid artery was cannulated for measurement of arterial pressure. In some animals a cannula with a 3-way stopcock was placed in the femoral vein for blood flow measurements. The venous outflow was measured before and during the infusions with a graded cylinder and stop watch. After a preliminary tissue fluid sampling and lymph collection the infusion of 10 μg/min or 2.5 μg/min histamine base or 0.4 μg/min bradykinin in physiological saline was started into the femoral artery. The rate of fluid infusion was 0.05 or 0.5 ml/min. The infusion was continued for the next 2h. One group of animals received 2 mg/kg phenoxybenzamine intravenously 30 min before the start of histamine infusion. Lymph collection in 30-min samples was continued during the infusion. To ensure a constant flow of lymph the extremities were passively moved by manually bending the leg in the knee joint with a frequency of 30/min. Tissue fluid was collected before and during the infusion. The first sample was obtained from the contralateral leg during the hour preceding the infusion and the second from the infused leg in the second hour of infusion. To this purpose 3 to 4 cotton wicks 4—6 cm long, previously soaked in physiological saline were sewn into the subcutaneous tissue of the shank and the paw. The wicks were pulled out after 1 h and the fluid expressed from them. Using this method about 0.02 to 0.05 ml of tissue fluid could be gained from each region. Lymph was collected in preweighed heparinized vials and its amount was determined gravimetrically. The circumference of the legs was measured below the knee joint at the beginning of the experiment and at its end. Each measurement was repeated 5 times and averaged.

Lactic acid dehydrogenase (LDH) was estimated in blood plasma, lymph and tissue fluid according to WROBLEVSZKY and LADUE [16], glutamic-oxalacetic transaminase (GOT) by the method of REITMAN and FRANKEL [11] and total protein according to LOWRY et al. [7]. The results presented in the text and Tables are means $\pm$ standard deviation of the means (S.D.). Statistical evaluation was made using the paired t test.

Results

Lymph flow

The average lymph flow in the 64 rabbits in the first 30 min after the cannulation of the prenodal leg lymphatic was 7.29 ± 1.07 mg/min. In the control group receiving only an intra-arterial infusion of 0.05 ml/min physiological saline lymph flow declined markedly during the 3h observation period. In the animals receiving 0.5 ml/min saline there was also a sharp decline after the first 30 min of the experiment. In the last hour lymph flow increased again, without, however, attaining the "control" level, i.e. the flow rate observed in the first half hour, before the start of the infusion. Histamine 2.5 or 10 μg/min introduced with 0.05 or 0.5 ml/min saline had only negligible effect on lymph flow rate. A clear-cut evidence for the effect of histamine on lymph flow was seen only in the animals receiving 2.5 μg/min histamine in 0.05 ml/min

Table I

Effect of histamine and bradykinin on crural lymph flow in the rabbit

	N	Infusion ml/min	Lymph flow mg/min					ΔC %	ΔP mm Hg
			—30—0	0—30	30—60	60—90	90—120		
Control	7	0.05	7.16	4.30	3.17	2.83	3.03	4.25	— 5.0
±S.D.			4.14	2.30	2.14	1.79	1.53	0.53	8.7
Histamine 2.5 μg/min	6	0.05	9.31	7.13	6.80	9.52	13.03	5.95	—10.0
±S.D.			1.28	1.77	0.63	2.33	3.15	0.12	8.7
Bradykinin 0.4 μg/min	7	0.05	7.39	10.01	12.10	15.82	18.87	9.95	—12.2
±S.D.			1.42	5.11	6.45	7.28	5.22	0.28	6.7
Control	8	0.5	8.16	5.82	4.98	6.92	6.87	5.35	— 8.6
±S.D.			3.69	2.79	2.49	4.23	3.12	0.24	9.0
Histamine 2.5 μg/min	7	0.5	8.88	7.09	6.71	6.67	6.67	4.90	—10.0
±S.D.			2.74	4.72	5.10	4.42	3.74	0.08	5.0
Histamine 10 μg/min	8	0.5	7.54	4.50	5.62	7.29	8.83	—0.25	—24.4
±S.D.			3.58	2.87	3.01	4.21	3.82	0.05	10.7
Phenoxybenzamine + Histamine	8	0.5	9.86	8.13	7.97	7.30	9.03		—32.2
10 μg/min ±S.D.			5.06	5.13	6.53	6.77	7.01		8.7
Bradykinin 0.4 μg/min	6	0.5	5.83	3.72	5.98	5.30	6.95	6.05	—24.2
±S.D.			1.81	1.93	4.71	3.25	3.41	0.18	9.7
Bradykinin 0.4 μg/min	7	0.5	6.11	8.48	17.37	27.77	33.67	11.35	—26.7
±S.D.			2.62	6.08	5.21	22.52	29.72	0.58	4.1

ΔC: Change of the circumference of the limb during the 2h infusion
ΔP Difference between systemic arterial pressures measured before the start and at the end of infusion

saline. Here in the control group lymph flow rate decreased from 7.2 to 3 mg/min while in the animals receiving histamine the flow rate, after an initial decrease, increased from 9.3 mg/min in the preinfusion period to 13 mg/min in the last half hour of histamine infusion (Table I). Pretreatment with phenoxybenzamine did not influence the response to histamine. Half of the animals (6 of 13) receiving 0.4 μg/min bradykinin in 0.5 ml/min saline did not respond with any appreciable change in lymph flow. In the other half of these animals there was, however, a very marked, about 5-fold increase of flow. The animals receiving the same bradykinin infusion but reacting in a different way were considered separately in the discussion. The lymph flow increased significantly ($p < 0.05$) also in the animals receiving bradykinin in 0.05 ml/min saline (from 7.4 mg/min to 18.9 mg/min).

Lymphatic and tissue fluid protein concentration

Similarly to previous observations [13, 14] total protein concentration was in the 64 animals in the control period, i.e. during the first hour of the experiments, significantly, by 18.7% higher in tissue fluid than in lymph. Total protein concentration in the lymph was 24.5 ± 2.2 and in the subcutaneous tissue fluid of the shank it was 29.1 ± 3.9 mg/ml. In the fluid collected from the paw protein concentration was markedly higher than in that obtained from the shank: 38.7 ± 9.9 mg/ml (Table II). In all animals including the control groups receiving only saline infusion protein concentration increased in the lymph and decreased in tissue fluid. Consequently, protein concentration in the tissue fluid sampled during the 2nd hour of infusion was lower than in the simultaneously collected lymph. This is not a consequence of the intra-arterial fluid infusion and/or fluid and protein loss from the cannulated lymph vessel. In 6 rabbits two tissue fluid samples were obtained, one in the first hour immediately after tying the animals to the operating table and the administration of the anaesthetic, and the second 2 h later. The animals did not receive infusion, the lymphatics were not cannulated and the leg was not moved as in the other groups. In this group tissue fluid protein concentration decreased during the 3 h of the experiment by 20%, from 26.0 ± 4.0 to 21.3 ± 3.9 mg/ml. This was probably a consequence of the dilution of the extracellular fluid. The circumference of the shanks increased during 3 h by 4% corresponding to an about 8% increase of volume. If this is due to fluid accumulation in the extracellular space it corresponds to an about 29% expansion of the extracellular fluid as a result of anaesthesia and the immobilization of the animals. Visible oedema was formed only in animals reacting with a significant increase in lymph flow. In these two groups the circumference of the shank increased by 10 and 11%, respectively. In the other groups the changes in cirumference did not differ from those observed in the controls. The only exception was

Table II

Effect of histamine and bradykinin on total protein concentration in tissue fluid and lymph (mg/ml)

	N	Infusion	Lymph					Tissue fluid				Plasma	
								Shank		Paw			
			—30—0	0—30	30—60	60—90	90—120	—60—0	60—120	60—0	60—120	—30	120
			minutes					minutes				minutes	
Control	7	0.05	25.5	28.3	28.0	28.8	28.1	23.8	19.4			61.0	56.2
S.D.			3.9	3.1	4.5	6.8	4.4	4.7	2.8			5.0	4.0
Histamine 2.5 μg/min	6	0.05	22.0	25.3	27.0	26.7	26.2	29.0	20.7			54.5	50.2
S.D.			0.4	2.8	4.9	2.0	3.0	1.3	1.5			3.5	5.1
Bradykinin 0.4 μg/min[a]	7	0.05	21.4	28.5	33.5	33.3	32.6	23.0	25.6			54.1	51.1
S.D.			3.1	7.9	6.6	5.7	5.5	4.1	8.3			5.5	6.5
Control	8	0.5	26.3	29.0	29.4	28.4	28.0	29.0	17.4			66.8	58.4
S.D.			2.0	3.9	5.7	6.9	6.8	5.3	3.7			7.6	7.2
Histamine 2.5 μg/min	7	0.5	24.6	25.2	29.8	30.5	29.3	34.8	28.1			64.5	55.2
S.D.			4.3	6.3	8.5	7.4	6.2	4.4	9.2			5.7	10.5
Histamine 10 μg/min	8	0.5	21.7	22.0	25.7	28.9	29.2	29.3	25.3			51.8	40.8
S.D.			4.3	4.0	3.0	3.3	3.3	3.1	3.4			4.3	3.1
Phenoxybenzamine + Histamine	8	0.5	25.5	23.7	24.8	24.8	21.9	30.7	19.3			64.6	55.0
10 μg/min, S.D.			4.9	1.7	3.8	4.0	4.1	6.3	3.7			9.8	6.8
Bradykinin 0.4 μg/min	6	0.5	26.9	27.6	29.7	31.6	32.4	30.7	23.9	38.8	30.5	59.7	45.3
S.D.			6.1	3.7	2.2	4.4	4.2	5.3	7.2	9.9	11.1	5.8	8.8
Bradykinin 0.4 μg/min[a]	7	0.5	26.9	30.0	35.4	34.4	31.1	31.2	27.0	38.4	28.7	59.7	44.0
S.D.			5.6	5.0	8.7	5.5	6.5	6.0	7.7	9.5	4.5	4.5	6.0
Anaesthesia + supine pos.	6							26.0	21.3			53.2	53.2
S.D.								4.0	3.9			7.8	6.5
	64		24.5					29.1				59.6	
			2.2					3.9				5.2	

[a] Animals with high lymph flow rates

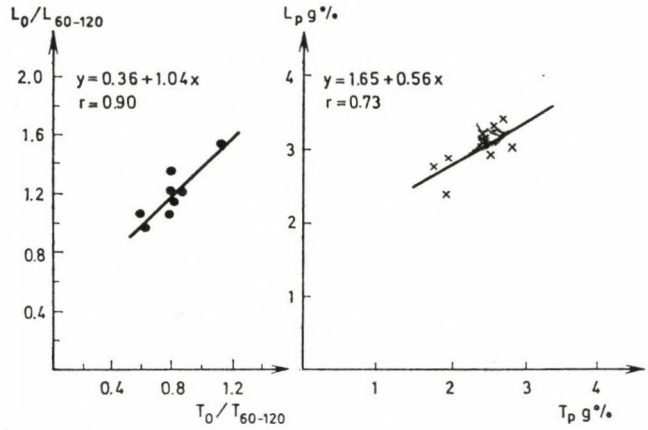

Fig. 1. A: Relationship between lymph and tissue fluid protein concentration during histamine or bradykinin infusion. The points represent the mean values for protein concentration in lymph and tissue fluid during the 2nd hour of infusion derived from Table II. B: Relationship between lymph and tissue fluid protein concentration changes. (Calculated from the mean values in Table II)

the group receiving 10 μg/min histamine where leg circumference did not increase. Lymphatic protein concentration was higher in the animals receiving bradykinin or histamine than in the controls. At the end of the experiment lymphatic protein concentration was augmented in the two control groups

Table III

Lymphatic protein fluxes
(μg/min)

	N	Infusion ml/min	Before infusion	During infusion
Control	7	0.05	183±12	85* ± 4
Histamine 2.5 μg/min	6	0.05	204±11	240+ ±76
Bradykinin 0.4 μg/min[a]	7	0.05	158±10	458*+ ±70
Control	6	0.5	215±28	172* ±21
Histamine 2.5 μg/min	7	0.5	218±25	194 ±11
Histamine 10 μg/min	8	0.5	164±15	203+ ±56
Phenoxybenzamine + Histamine 10 μg/min	8	0.5	214±25	194 ±10
Bradykinin 0.4 μg/min	6	0.5	160±22	190*+ ±30
Bradykinin 0.4 μg/min[a]	7	0.5	164±15	718*+ ±180
	62		186±26	

* Significant change ($p < 0.05$) compared to the preinfusion period
\+ Significant change ($p < 0.05$) compared to the control group
[a] Animals with high lymph flow rates

by 11 and 8%, respectively. In the two groups where bradykinin increased lymph flow lymphatic protein concentration was raised by 34% and 54%, while in the group where it did not influence the flow the change was only 17.5%. Finally, in all three groups receiving histamine infusion protein concentration increased by about 22%. No change or a slight decrease of lymphatic protein content was seen only in animals pretreated with phenoxybenzamine before histamine administration.

The lymphatic protein fluxes which declined in the controls as a result of diminished lymph flow increased in almost all animals receiving bradykinin or histamine. Marked changes were seen in the bradykinin infused animals with high lymph flow rates. In the two groups lymphatic protein transport increased 3 and 5 times, respectively. In previous investigation under normal conditions and at increased venous pressure and/or hypoproteinaemia a high correlation was detected between tissue fluid and lymph protein concentrations. A similar correlation was detected also during bradykinin or histamine infusion. There was also a strong correlation ($r = 0.90$) between the changes of tissue fluid and lymph protein concentration (Fig. 1).

Enzyme activities in tissue fluid and lymph

As it was established previously [12, 14, 15] LDH activity is much higher in tissue fluid than in regional lymph. In the present study in 64 rabbits LDH activity in the crural lymph was 181 ± 48 mU/ml and in the tissue fluid of the shank 280 ± 40 mU/ml. The tissue fluid obtained from the paw of the animals contained 432 mU/ml LDH (Table IV). During the 3h of the experiment in the control animals similar changes could be detected in LDH activities as it was observed for total protein: a slight increase in lymph and decrease in tissue fluid. Due to the decrease in lymph flow rate lymphatic fluxes were slightly decreased or unchanged. Both histamine and bradykinin increased lymphatic LDH transport in all experimental groups and consequently the release of the enzyme from the cells (Table V). In the experimental groups where histamine or bradykinin did not increase significantly lymph flow lymphatic LDH concentration increased significantly in face of no or only a moderate change in tissue fluid LDH activity. In the two groups with greatly increased lymph flow total LDH transport by the lymph increased about 2 to 3 times while lymphatic concentrations decreased markedly. This is obviously a dilutional effect, because there was a strong negative correlation between lymph flow rate and lymphatic LDH activity (Fig. 2). The results concerning the effect of histamine and bradykinin on GOT activities in plasma and lymph were inconclusive (Table VI). The only significant change was the increase in tissue fluid and lymph GOT during the 10 μg/min infusion of histamine.

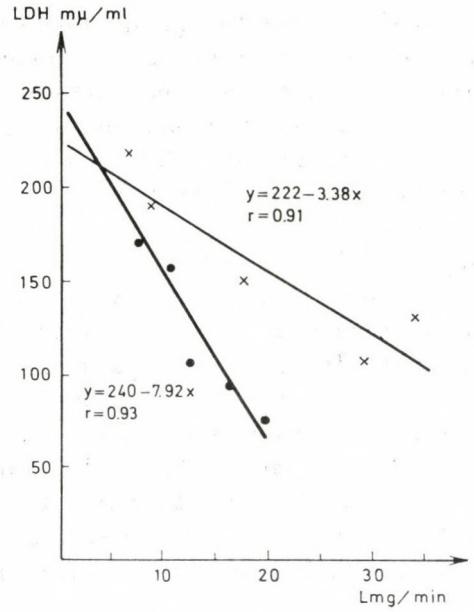

Fig. 2. Correlation between lymph flow and lymph LDH in the two groups of animals reacting with an increased lymph flow rate to bradykinin infusion. (The points in the Figure are mean values for the individual periods from Tables I and IV)

Discussion

Histamine and occasionally bradykinin too failed to increase lymph flow and did not lead to oedema formation in rabbits. In the animals where histamine or bradykinin did not increase lymph flow the circumference changes of the limbs were not greater than in the controls. On the other hand, a larger dose of histamine produced a small, not significant decrease in hindlimb circumference. In dogs and cats a close arterial infusion of the two agents into the extremities leads to oedema formation, increased lymph flow rate and lymphatic protein concentration [3, 4, 5, 6, 11]. It has been suggested that these effects are due to increased microvascular pressure as well as to a pressure independent action [2]. The latter seems to be the increased microvascular permeability [14]. The changes were associated with large increases in blood flow and small vein pressure. When histamine was infused intravenously in dogs it decreased forelimb vascular pressure and blood flow. Forelimb weight decreased falling well below control by the end of the 4h infusion period [1, 9]. In the present study in rabbits, small doses (2.5 μg/min) of histamine failed to influence blood flow. During a 5—10 min infusion of 10 μg/min histamine femoral venous flow decreased, however, from 5.3 ± 2.5 to 3.7 ± 2.1 ml/min. Systemic arterial pressure dropped in these animals by

Table IV

Effect of histamine and bradykinin on LDH *activities in tissue fluid and lymph* (mU/ml±S.D.)

	N	Infusion ml/min	Lymph					Tissue fluid				Plasma	
								Shank		Paw			
			—30—0	0—30	30—60	60—90	90—120	—60—0	60—120	—60—0	60—120	—30	120
			minutes					minutes				minutes	
Control	7	0.05	212	259	249	251	258	301	259			48	38
S.D.			115	102	74	95	110	78	92			17	16
Histamine 2.5 μg/min	6	0.05	297	278	298	259	321	395	338			45	36
S.D.			70	70	126	92	101	29	81			27	15
Bradykinin 0.4 μg/min[a]	7	0.05	172	156	105	94	85	357	378			57	40
S.D.			117	97	43	45	40	247	236			35	14
Control	6	0.5	129	171	150	140	152	250	235			39	23
S.D.			40	65	51	52	50	95	119			9	6
Histamine 2.5 μg/min	7	0.5	153	124	158	203	327	296	292			37	27
S.D.			44	49	38	63	210	102	95			9	10
Histamine 10 μg/min	8	0.5	172	154	284	486	700	207	313			29	38
S.D.			82	58	126	229	98	68	105			14	16
Phenoxybenzamine + Histamine	8	0.5	135	169	175	192	208	271	293	414	387	40	30
S.D.			72	93	93	111	96	130	109	106	203	16	14
Bradykinin 0.4 μg/min	6	0.5	239	191	296	345	416	268	284	438	445	44	40
S.D.			156	155	177	184	177	157	158	81	132	20	20
Bradykinin 0.4 μg/min[a]	7	0.5	217	189	150	107	130	291	207	426	207	44	40
S.D.			90	95	38	58	108	120	94	81	91	20	20
	64		181					280				39	
			49					40				11	

[a] Animals with high lymph flow rates

Table V

Lymphatic LDH fluxes
mU/min

	N	Infusion ml/min	—30—0	90—120 min
Control	7	0.05	1.45±0.48	0.78* ±0.17
Histamine 2.5 μg/min	6	0.05	2.29±0.07	4.17**±0.30
Bradykinin 0.4 μg/min[a]	7	0.05	1.39±0.17	2.89**±0.21
Control	6	0.5	0.99±0.15	1.04 ±0.16
Histamine 2.5 μg/min	7	0.5	1.36±0.12	2.18* ±0.78
Histamine 10 μg/min	8	0.5	1.30±0.29	6.18**±0.37
Phenoxybenzamine + Histamine 10 μg/min	8	0.5	1.33±0.36	1.88* ±0.67
Bradykinin 0.4 μg/min	6	0.5	1.27±0.28	1.60* ±0.60
Bradykinin 0.4 μg/min[a]	7	0.5	1.32±0.23	4.37**±0.32
	62		1.32±0.15	

Significant changes compared to preinfusion period * $p < 0.05$
** $p < 0.01—0.001$

[a] Animals with high lymph flow rates

24.4±10.7 mmHg and there was no significant change in local vascular resistance. Accordingly, the decrease of flow was largely due to the diminished driving force. Hypotension is usually associated with an increased reflex sympathoadrenal discharge and histamine might also cause a direct release of catecholamines [9]. When histamine was infused simultaneously with catecholamines into the dog brachial artery, oedema formation and increased lymph flow were greatly reduced and lymphatic protein concentration did not increase significantly. Phentolamine failed to prevent the antagonism of catecholamines and histamine [10]. In the present study sympathetic α-receptor blockade by phenoxybenzamine did not influence the action of histamine. This is consistent with the assumption that catecholamines antagonize the action of locally infused histamine independent of α-receptor activity [10].

Bradykinin infused into canine forelimbs perfused at constant inflow with autologous blood from haemorrhaged animals failed to increase lymph protein concentration or to produce visible signs of oedema formation [8]. This antagonistic effect was attributed to substance(s) in the blood liberated by haemorrhage which antagonized the action of bradykinin on the microvascular membrane. It was actually shown that some vasoactive agents, e.g. catecholamines [14], vasopressin, serotonine, methylprednisolone [12] possess the unique ability to prevent the increase in protein efflux by bradykinin. The lack of action of bradykinin observed in some animals similarly as in the

Table VI

Effect of histamine and bradykinin on GOT activities in lymph and tissue fluid

	N	Infusion ml/min	Lymph					Tissue fluid Shank		Plasma	
			—30—0	0—30	30—60	60—90	90—120	—60—0	60—120	—30	120
			minutes					min		min	
Control	7	0.05	36	34	36	38	46	20	32	21	26
S.D.			17	12	13	11	16	14	16	12	16
Histamine 2.5 μg	6	0.05	23	23	28	29	29	25	36	19	27
S.D.			15	16	15	15	13	13	11	13	16
Bradykinin 0.4 μg	7	0.05	18	16	16	16	18	19	28	13	16
S.D.			14	6	7	8	9	4	11	4	7
Control	6	0.5	14	16	15	13	15	15	19	9	12
S.D.			9	10	9	5	12	6	6	7	9
Histamine 2.5 μg	7	0.5	17	12	13	11	16	14	16	12	16
S.D.			10	8	9	9	14	7	8	9	9
Histamine 10 μg	8	0.5	15	16	16	21	30*	16	25*	17	21
S.D.			7	9	10	14	13	8	9	4	7
Phenoxybenzamine + Histamine 10 μg	8	0.5	26	20	27	27	30	21	27	20	26
S.D.			16	13	18	19	17	12	24	15	21
Bradykinin 0.4 μg	6	0.5	33	37	34	32	30	33	37	23	28
S.D.			23	13	19	30	26	26	20	14	18
Bradykinin 0.4 μg	7	0.5	20	24	24	23	28	31	35	19	25
S.D*t*			14	20	18	17	23	19	25	15	22

* Significant difference ($p < 0.05$) compared to the preinfusion samples

histamine infusion experiments might be due to some of the above substances being liberated as a result of hypotension induced by infusion of the two agents.

On the other hand, the observations support our previously expressed opinion that subcutaneous tissue fluid consists at least of two compartments. Under normal conditions the concentration of macromolecules, both of tissue origin (LDH) and of those escaping from the microvasculature (plasma proteins), is higher in tissue fluid than in lymph. A diffuse tissue injury induced by ischaemia or burning leads to a massive release of cellular proteins including intracellular enzymes. The best indicator of this process is the change of LDH activity in tissue fluid and lymph [18]. After 4 h of limb ischaemia LDH activity in the regional lymph increased, e.g. from 200 ± 48 to $3900 \pm \pm 1870$ mU/ml, at the same time tissue fluid activity increased from 400 ± 40 to $36{,}300 \pm 11{,}500$ mU/ml [18]. Thermal or ischaemic trauma leads also to vascular injury. This is reflected by the increased protein concentration both in tissue fluid and lymph and the disappearance of the difference in protein concentration between the two fluids [17, 18]. In the present experiments intra-arterially infused bradykinin and histamine failed to cause diffuse tissue injury. Tissue fluid LDH activity, with the exception of a single experimental group, did not change significantly. On the other hand, in all 4 experiments where bradykinin and histamine failed to affect significantly lymph flow there was a marked increase in lymphatic LDH. The greatest change was observed in animals receiving 10 μg/min histamine. LDH in the lymph increased from 170 to 700 mU/ml and in this group there was also a minor increase in tissue fluid LDH: from 207 to 303 mU/ml. The release of LDH into the lymph was markedly augmented also in animals where bradykinin produced an increase in lymph flow rate, however, in these animals LDH in the lymph was diluted by the fluid escaping from the circulation, its concentration consequently becoming significantly lower than before bradykinin administration. At the same time LDH in the tissue fluid samples was not or only moderately diluted. It is concluded that the vasoactive agents leaving the microvessels induce a cell injury localized at the perivascular interstitial space. The results suggest that only this perivascular interstitial fluid is drained directly by the lymph vessels and that lymph represents essentially recent microvascular filtrate. The second compartment of the interstitial fluid rinsing the cells and the connective tissue fibres is parallelly coupled with the first and seems to be in exchange with it. This conclusion is supported also by the oppositely directed changes in tissue fluid and lymph protein concentrations. On the other hand, the correlation between lymphatic and tissue fluid concentration changes suggests a ready exchange between the two compartments. The interstitial matrix seems, however, to offer a substantial resistance to bulk flow and to macromolecular diffusion. This is reflected by the concentra-

tion gradient of proteins of cellular origin which may be quite considerable if their release is enhanced by tissue injury or by the action of vasoactive substances and by the lack of concentration equilibrium of extravascular plasma proteins between tissue fluid and lymph.

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EFFECT OF SYMPATHOMIMETIC DRUGS (EPINEPHRINE, DOPAMINE, ISOPROTERENOL AND EPHEDRINE) ON GLUCOSE CONSUMPTION AND GLYCOGEN CONTENT OF *TETRAHYMENA*

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Epinephrine, dopamine and isoproterenol (10^{-3} M) stimulated glucose consumption of *Tetrahymena* of the GL strain, while ephedrine was without effect. Epinephrine (10^{-6} M) increased glycogen content in the absence of exogenous glucose, the other drugs not having such an effect. However, in the presence of glucose, glycogen content was increased to the same extent after addition of the drugs with the exception of epinephrine, the effect of which was less pronounced.

The protozoon *Tetrahymena* contains several hormones [1, 14, 16, 17] and it responds to several other hormones as well which are present in higher organisms only [2, 3]. Its glucose metabolism is influenced by epinephrine [7] and insulin [6] both of which are present in this protozoon, the phagocytotic ability is influenced by histamine and serotonin [8], the latter substance being present in cell division is affected by thyroxine and its precursors [10], and RNA synthesis is influenced by steroid hormones [11], by polypeptide hormones of the hypophysis [12], and by various plant hormones as well [13]. The ability of the receptors to select seems to be very specific, although overlappings do occur. This organism can differentiate histamine and serotonin from its derivatives when assessed by measuring phagocytosis index [9] and thyroxin from its precursors as shown by determination of cell division [10].

The present experiments were designed to study the effect of epinephrine and of other sympathomimetic drugs on the glucose metabolism of *Tetrahymena*, and to obtain information about the differentiating ability of the receptors in this organism.

Materials and Methods

Two days-old-(exponential phase) *Tetrahymena pyriformis* cultures from the GL strain, incubated in 1% trypton (Difco, Michigan) and 1‰ yeast extract at 28 °C, were used in the experiments.

In order to starve the cultures they were kept in Losina physiological salt solution for 24 hours prior to the experiments.

1. *Detection of glucose metabolism*

Tetrahymena cell suspensions were placed into glucose solution (final glucose concentration: 100 mg per 100 ml) diluted by Losina solution in the absence (control) or in the presence of increasing concentrations (10^{-5}—10^{-4}—10^{-3} M) of sympathomimetic drugs (treated groups).

The drugs used were as follows: dopamine (Fluka, Switzerland); isoproterenol (Fluka, Switzerland); epinephrine (Richter, Budapest) and ephedrine (Chinoin, Budapest).

Hormone and glucose were added simultaneusly for 10 minutes. The experiments were carried out in groups consisting of 10 cultures and they were repeated four times. Thus 40 measurements were made in all experimental groups. Deproteinization was carried out by perchloric acid after hormone, or glucose treatment and the glucose content of the supernatant was determined spectrophotometrically by the Boehringer (Mannheim) GOD-Perid method. Glucose consumption was calculated for one *Tetrahymena*.

2. *Measurement of changes in glycogen content*

The experiments were carried out similarly to that described above with the exception that there were always cultures in the hormone-treated groups which were not treated with glucose. In these cultures we studied the effect of the hormones (in Losina solution) on the amount of PAS positive material. A further difference was that hormones and glucose were not added at the same time in this series, but the 10-min glucose treatment followed hormone treatment by 20 to 30 or 40 min. *Tetrahymena* were thereafter washed out from the solution, they were fixed in Carnoy solution and the PAS reaction was carried out the intensity of which as well as its amount in the cell was determined by a Zeiss Amplival cytophotometer at 434 nm in the scanning mode. Thirty *Tetrahymena* were investigated in all experimental groups. The results are expressed in cytophotometric (arbitoiry) units, which was standard in the whole experimental series.

Statistical significance of the results was estimated by the Student's t test in both experimental series.

Results and Discussion

Significant effect on glucose consumption of *Tetrahymena* was achieved by the drugs at a concentration of 10^{-3} M (Fig. 1). Lower concentrations of the drugs were also effective, but they were not statistically significant (the effective concentration is very high as far as the mammals are concerned, however, the mammalian doses are not valid for the *Tetrahymena*). After epinephrine, dopamine and isoproterenol treatment *Tetrahymena* consumed 8 to 10 times more glucose than under control conditions, while ephedrine caused only mild elevation of glucose uptake which did not prove to be statistically significant. These results agree well with previous data obtained in *Planaria* [4], where epinephrine and dopamine proved to be the most potent and ephedrine the less active sympathomimetic agent. As far as the difference between the action of epinephrine and ephedrine is concerned, they are similar to the sequence observed in the human [15]. It may be assumed that the epinephrine precursor dopamine is a "more important hormone" than epinephrine itself on the low level of phylogenesis. This assumption has been put forward in previous experiments [4].

Glucose consumption was significantly influenced by the highest drug concentration only, while the lowest hormone concentration (10^{-6} M) exerted a statistically significant effect on the amount of PAS positive material. The

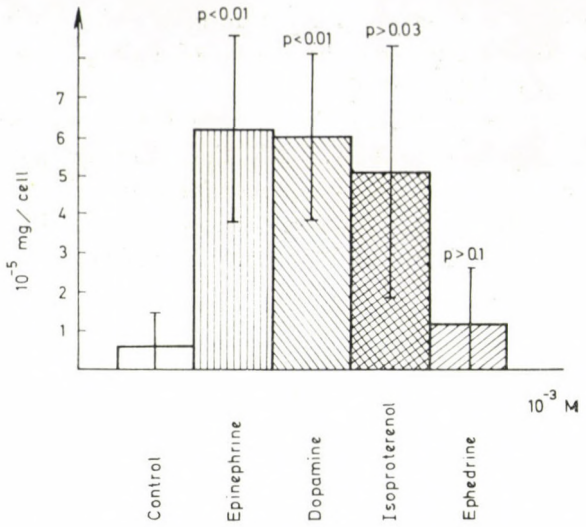

Fig. 1. Glucose consumption of *Tetrahymena* in the presence of 10^{-3} M sympathomimetic drugs

possibility exists that the sensitivity of the two methods differs considerably, or also that the hormones in physiological concentration influence primarily *endogenous* process rather than exogenous glucose uptake itself. This suggestion is confirmed by the present finding that the glycogen contents of *Tetrahymena* incubated in the absence of exogenous glucose increased significantly after epinephrine treatment for 20 min indicating endogenous glycogen synthesis from endogenous glucose sources (Fig. 2).

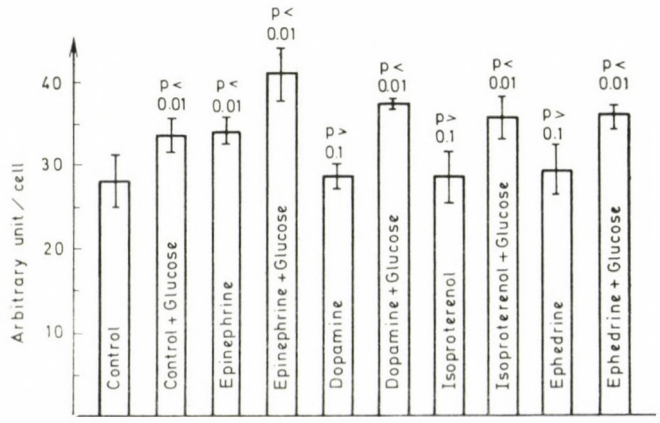

Fig. 2. The change of glycogen content of *Tetrahymena* after 20-min treatment by sympathomimetic drugs in the presence and absence of exogenous glucose. (The mean value of PAS positive material is expressed for one animal)

The fact that the effect of epinephrine in vertebrates is opposite is not astonishing since in *Tetrahymena* the effects of epinephrine and insulin on glucose metabolism are similar [9].

Remarkably enough during the 20-min treatment, only epinephrine increased cellular glycogen content, while the other sympathomimetic analogues were ineffective. At the same time, these derivatives increased intracellular glycogen content in the presence of exogenous glucose, although to a much smaller extent than epinephrine, and there was no difference between the effects of ephedrine and that of the other drugs.

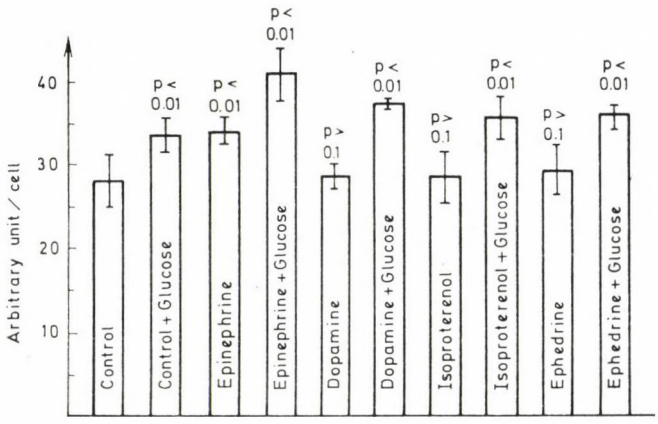

Fig. 3. The change of glycogen content of *Tetrahymena* after 30-min treatment by sampathomimetic drugs in the presence or absence of exogenous glucose. (The mean value of the amount of PAS positive material is expressed for one animal)

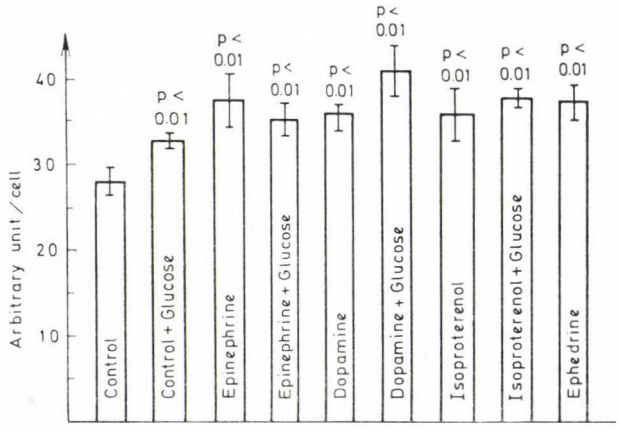

Fig. 4. The change of glycogen content of *Tetrahymena* after 40-min treatment by sympathomimetic drugs in the presence or absence of exogenous glucose. (The mean value of the amount of PAS positive material is expressed for one animal)

Since epinephrine is present in the *Tetrahymena* under normal conditions [1, 16] it is possible that this hormone regulates glycogen accumulation. This means that exogenous epinephrine does not act on the membrane receptors of *Tetrahymena* but rather penetrates into the cell and increases intracellular glycogen content in such a way. However, all other drugs are foreign materials and they can not exert such an intracellular action, however, they stimulate glucose consumption (uptake) by interaction with membrane receptors, which leads to an increase of intracellular glycogen content (epinephrine was also able to stimulate glucose uptake). This suggestion is confirmed by the present finding that during long (30 to 40 min) treatment with epinephrine analogues the glycogen content increased, while at the same time such a long lasting treatment with epinephrine caused a decrease of glycogen accumulation (Figs 3 and 4). It is possible that the analogues, as foreign substances, need longer time for the stimulation of glycogen synthesis than epinephrine does.

In conclusion the present experiments demonstrate that epinephrine stimulates both glucose consumption and glycogen accumulation in the *Tetrahymena*. Glucose uptake is stimulated only by high hormone concentrations, while glycogen accumulation is increased by lower concentrations as well.

As far as glucose consumption is concerned *Tetrahymena* can not differentiate between epinephrine, dopamine and isopreterenol, but the organism is insensitive to ephedrine. However, there are large differences between the effects of epinephrine and its analogues as far as the endogenous glucose–glycogen transformation is concerned, this differences being much less pronounced if exogenous glucose is also present.

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ASSUMED ROLE OF L-ARGININE IN MOBILIZATION OF ENDOGENOUS FORMALDEHYDE

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It is assumed that the normal formaldehyde equilibrium in the organism is governed by a biological control mechanism, which is thought to be closely connected with l-arginine and indirectly with the arginase enzyme. Arginine reacts with formaldehyde in a spontaneous equilibrium reaction yielding methylol derivatives, thus mobilizing formaldehyde. The methylol derivatives of arginine were found in serum and urine; they seem to have an inhibitory effect on cell proliferation.

In biological systems, N^{ε}-methylated lysine derivatives may be present in free and bound forms [2, 12]. Methyl groups come partly from methionine [1, 11]. In the presence of methionine adenosyl transferase (EC 2.5.1.6.) and ATP, L-methionine is transformed into S-adenosyl-methionine which contains a high energy methyl-sulphonium group. The latter compound is able to methylate lysine in the presence of methyltransferases. At the same time, S-adenosyl-methionine, after losing a methyl group, is transformed into S-adenosyl-homocysteine. Though methionine plays a fundamental role in methylation processes, it is formed by methylation of homocysteine. The methylating agent in this reaction is N^5-methyl-tetrahydrofolate.

In biological systems a "pool" of C_1 methyl groups has a basic significance. N^6-methyl-lysine-oxidase catalyse the decomposition of N^{ε}-monomethyl-L-lysine to L-lysine and formaldehyde. The resulting endogenous formaldehyde, if it is in excess, may hypermethylate nucleinic acids and histonic proteins beside the direct enzymatic transmethylation. Several concepts are known in connection of biological methylation and malignant cell proliferation [15, 4, 9]. Formation of endogenous formaldehyde, which can have a purely chemical, enzymatically not supported, methylating effect, can take place from nitrosamines as well. This reaction is catalysed by N-demethylase [16, 3]. Environmental factors like viral infections also have an influence on the formation of formaldehyde *in vivo* [5].

It seems that there exists some mechanism that controls the enzymatic and chemical (*via* formaldehyde) methylation processes so that these remain within normal limits. Hypermethylation can take place because of some defect

in the controlling system. There seems to be a mechanism that prevents the accumulation of endogenous formaldehyde. This mechanism works so that the undesirable surplus of formaldehyde should react with some formaldehyde-acceptor and is eliminated after having been changed structurally due to this interaction. It is supposed that in this respect first arginine and then certain

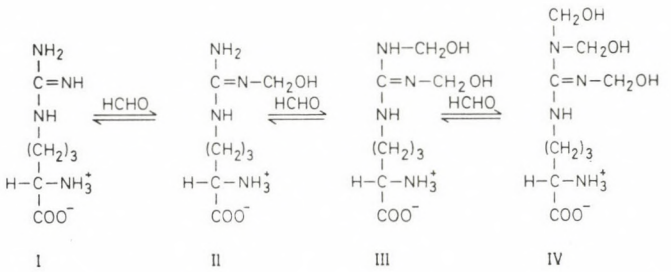

Fig. 1. Scheme of the hydroxymethylation reaction between L-arginine and formaldehyde

mobile compounds, e.g. cysteine or glutathione containing thiol groups, and the formaldehyde-reducing ascorbinic acid should be taken into consideration. The guanidino groups of arginine react rapidly with formaldehyde. The rate of this reaction is higher than that of any other reaction of formaldehyde taking place in biological systems. The ability of arginine to bind formaldehyde makes it possible to mobilize formaldehyde in the form connected to arginine. The product of this interaction is the methylol derivative of arginine. The methylol structure is a weak chemical bond, that is easily split by environmental changes to arginine and formaldehyde [13].

The experimental section of the present paper deals with the hydroxymethylation reaction between arginine and formaldehyde. The arginine shows great affinity towards formaldehyde. A general idea of how this spontaneous reaction takes place is given in Fig. 1.

We believe that hydroxymethylation takes place first on the imino part of the guanidino group and then on the primary amino group. The ratio of the products depends on pH; the less it is, the more the equilibrium shifts to the left. The methylol structure was proven by NMR — spectroscopy. The methylol derivatives of arginine were detected from both urine and blood [14].

In our experiments the rate of reaction was studied under physiologic conditions of pH and ionic strength, at different initial concentrations.

The rate constant and the order of the reaction were calculated.

Materials and Methods

Materials used:

- L-arginine, a.lt. (Reanal, Budapest)
- formaldehyde, 35% aqueous solution (Reanal, Budapest)
- Sephadex G-15 (Pharmacia, Uppsala)
- phosphate-buffer according to Sörensen pH 7.34; 1.82 g KH_2PO_4 diluted to 1000 ml in 9.50 g $Na_2HPO_4 \cdot 2\,H_2O$ distilled water.

Formation of the methylol derivatives of arginine was studied using the differential spectrophotometric method with a deuterium lamp as light source at a wave length of 220 nm. This method was chosen because it allowed to study the course of the reaction while other methods, which determine the amount of the residue, e.g. specific reagents for formaldehyde or the guanidino group, give indirect results.

Results

First, a mixture of mono, di- and trimethylol arginine was prepared. 3.0 mM arginine was dissolved in 4.0 ml of a solution of 5% formaldehyde and 95% buffer and the mixture was kept in a closed vessel at 27 °C for 24 hours.

Then 3.0 ml of the mixture was applied to a Sephadex G-15 column equilibrated with distilled water and was eluted with distilled water. 10 ml fractions were collected and analysed spectrophotometrically. Spectrally responsive fractions appeared from 70 ml on. The first compounds to come down from the column were tri-, di- and monomethylol arginine, which have molecular weights greater than that of arginine. It was known from preparatory tests that arginine was to appear in the 12th while formaldehyde in the 17th fraction. Thus fractions 7—11 were combined and dried at room temperature to yield a white crystalline mixture of mono-, di- and trimethylol arginine.

Data concerning reaction kinetics of the hydroxymethylation of arginine

The reaction can be examined quantitatively by differential spectrophotometry. Examinations were carried out under physiological conditions (pH 7.38; room temperature). Reaction mixtures contained formaldehyde in excess; the absolute concentration was 0.5%. As formaldehyde can not be characterized spectrally in the UV range, the first step concerning kinetics was to measure the relative extinction of a 1:1 mixture of 1.0 mmol/l arginine and 1.0% formaldehyde solutions against a reference arginine solution of 0.5 mmol/l concentration and to examine the change of extinction during the reaction.

The graphs in Fig. 2 suggest that absorbancy is proportional to concentration at the wave length of 220 nm. As formaldehyde was in excess in the system, the rate of the reaction can be modelled with first order kinetics.

To determine the rate constant, four reaction mixtures 4.0 ml each of different initial concentrations were studied at room temperature. The initial

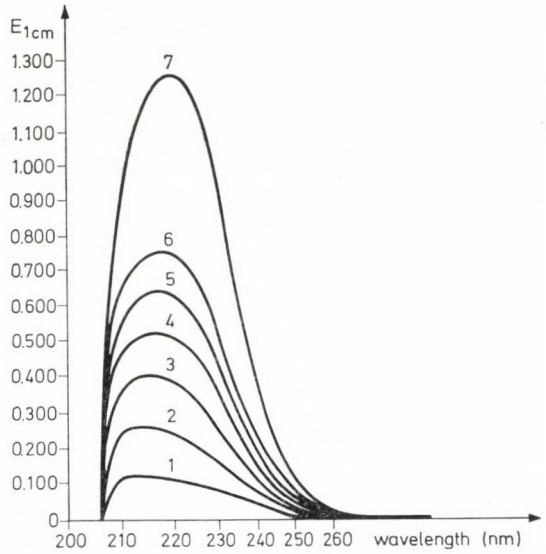

Fig. 2. Spectral tracking of the formation of methylol-arginine. Conditions: room temperature, pH 7.38; concentration of arginine = 0.5 mmol/l. Specord UV—VIS (Zeiss) type spectrophotometer. 1. 4 min; 2. 8 min; 3. 12 min; 4. 16 min; 5. 20 min; 6. 24 min; 7. 60 min

concentrations of arginine and formaldehyde were 1.0—166.7; 0.75—166.7; 0.50—166.7 and 0.25—166.7 mmol/l, respectively. The value of 166.7 mmol/l corresponds to 0.5%. Concentrations of arginine in the reference samples were equal to the initial concentrations of arginine in the four reaction mixtures.

Figure 3 shows the change of extinction during the reactions corresponding to different initial arginine concentrations.

It can be seen from Fig. 3, that the higher the original concentration, the more steeply the curves start. This means that the rate of reaction is proportional to the concentration of arginine.

The rate constant of a first order reaction is given by the expression,

$$k = \frac{1}{t} \ln \frac{(A)_0}{(A)_0 - X} \; ; \quad (1), \text{ where in our case}$$

k = rate constant (min^{-1});
$(A)_0$ = initial concentration of arginine, mmol/l;
X = concentration of arginine at "t" time, mmol/l;
t = time of the reaction, min.

The above formula can be transformed as follows. If E_0 represents the relative extinction of the initial reaction mixture and E_∞ represents the relative extinction when the reaction is complete then E_0—E_∞ is proportional to the

initial concentration, i.e. $(A)_0 = b(E_0 — E_\infty)$. This is how much the relative extinction changes before the hydroxymethylation had reached equilibrium. In the above equation b = constant. If, at a particular "t" time, the relative extinction of the solution is E, then the concentration of arginine reacted so far can be expressed as: $X = b(E_0 — E)$.

Substituting the above expressions into (1) we get

$$k = \frac{1}{t} \ln \frac{E_0 - E_\infty}{E - E_\infty}; \quad (2), \text{ where}$$

k = rate constant (min^{-1});
E_0 = relative extinction at $t = 0$ (zero in our case);
E_∞ = extinction(s) in equilibrium;
E = extinction(s) at "t" time;
t = time of reaction (min^{-1}).

On the basis of equation (2), reaction rate constants corresponding to 0.25 and 0.50 mmol/l concentrations of arginine were determined graphically from curves 1 and 2, of Fig. 3 (where equilibrium is represented by horizontally ending curves).

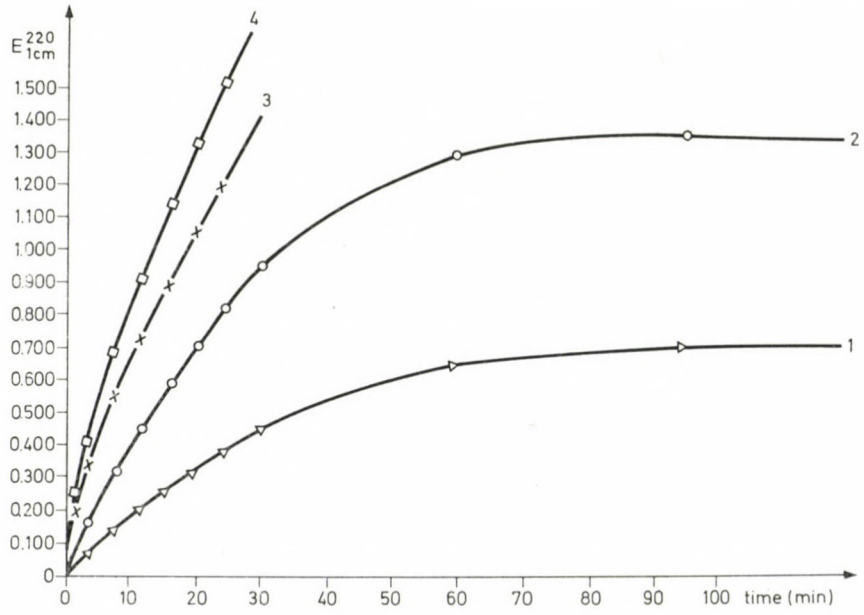

Fig. 3. Time dependence of hydroxymethylation of arginine at different initial concentrations. Specord UV—VIS (Zeiss) spectrophotometer. Wave length: 220 nm. Concentrations of arginine: 1. 0.25 mmol/l; 2. 0.50 mmol/l; 3. 0.75 mmol/l; 4. 1.00 mmol/l

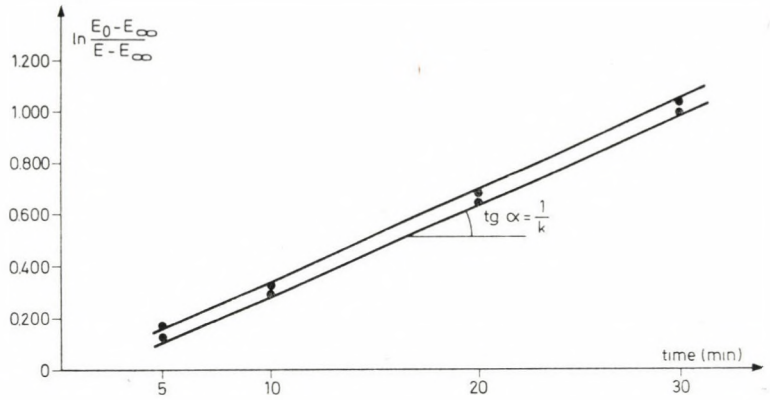

Fig. 4. Diagram used to determine the rate constant. Data derived from curves 1 and 2 of Fig. 3

$\ln \frac{E_0 - E_\infty}{E - E_\infty}$ values were calculated and plotted against time to yield straight line(s). The rate constant was determined from the slope of the line(s). The fact of linearity is another evidence of the first order reaction (Fig. 4). $\operatorname{tg} \alpha$ of the above line(s) is: $\operatorname{tg} \alpha = \frac{1}{k} = 0.035$; and the calculated rate constant: $k = 28.57\ \text{min}^{-1}$.

Discussion

Our concept of the mechanism of the hydroxymethylation of arginine is supported by the fact that its aqueous solution is neutral. This is only possible if the twin ion form of the molecular structure remains unchanged, i.e. there is no free primary amine on the guanidino group; otherwise the solution would be basic. The fact that the α-amino group remained intact was shown us by the positivity of the ninhydrine test. Formaldehyde, mobilized in the form of hydroxymethylated arginine which is present in both blood and urine, can have access to cell components it could not attain spontaneously. In this respect methylol-arginine can be regarded as a formaldehyde donor. The methylol-derivative of arginine can thus bring formaldehyde into a biologically effective state (biophase) that it otherwise could not reach, being hindered by various barriers. Under given ionic strength and pH conditions, the methylol derivative is likely to be in equilibrium with the concentration of intact arginine and free formaldehyde in the system. In our model, the endogenous or exogenous accumulation of arginine in a biological system would result in a decrease of the free endogenous formaldehyde concentration or in an increase of the methylol-derivative of arginine. A decrease of arginine would cause the amount

of free endogenous formaldehyde to increase or that of the methylol derivative to decrease. In another interpretation this means that the increase of endogenous formaldehyde due to some exogenous reason will lead to a decrease of the free arginine level and thus a shift in the arginine equilibrium. In this respect arginine, an amino acid that eliminates formaldehyde, can be regarded as a defensive agent. If the amount of arginine mobilized from the reserves

Table I

Effect of different materials on the activity of arginase and methionine-adenosyl transferase

	Inhibitors	Activators	Reference
Arginase E. C. 3.5.3.1.	L-ornithine; Zn^{2+}; Hg^{2+}; L-lysine; Ag^{+}; citrate	$Mn^{2+} > Co^{2+} > Fe^{2+}$	(7)
Methionine adenosyltransferase E. C. 2.5.1.6.	K^{+}; NH_4^{+}; Rb^{+}	$Mg^{2+} > Mn^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+}$	(8)

is insufficient to eliminate the undesirable surplus of formaldehyde, the accumulation of endogenous formaldehyde will result in hypermethylation.

Summarizing the above findings, the control of endogenous arginine can ensure a biological equilibrium, the desirable level of endogenous formaldehyde.

As to over-, and hypermethylation, we suppose that its control is limited to the control of arginine, and indirectly to the control of arginase enzyme and enzymatic transmethylation by methionine adenosyltransferase. Taking this into consideration we may state that hypermethylation will be avoided if the two enzymes in question are inhibited.

Some of the known activators and inhibitors of arginase and methionine adenosyltransferase are listed in Table I.

In *in vivo* systems, inhibition of enzymatic methylation is not advisable because of the side effects of the inhibiting monovalent ions. As to arginase, excessive amounts of its activators cause the arginine equilibrium to shift to lower arginine levels. If excessive amounts of Mn^{2+}, Co^{2+}, Fe^{2+} and Mg^{2+} ions reach the organism from foods and fluids, they may increase methylation to an undesirable extent. The effect can be counterbalanced by Zn^{2+}, citrate ions and lysine.

Thus if the ratios of the above ions and the two amino acids lysine and arginine are unfavourably shifted, the equilibrium of the system will be upset and hypermethylation will take place. As the global amount of metal ions can be decreased by complex forming substances, moderate dosage of certain complex forming substances might help to restore the equilibrium of the corresponding metal ions.

Another possible conclusion is that an inhibition of arginase slows down indirectly the operation of the ornithine decarboxylase shunt which originates in the urea cycle. Polyamines are known to play a significant role in the development of certain kinds of tumour [6]. Inhibition of arginase neight result in a decrease of the amount of these polyamines.

Formaldehyde which is always present in blood and urine, was observed to disappear in the final phase of cancer [10]. This is probably a consequence but if it were a cause, then formaldehyde would have access to the organism in the form of neutral methylol-arginine. Taking into consideration the fact that arginine derivatives are complementary substances to the cell proliferation stimulating trimethyl-lysine, methylol-arginines may be regarded as inhibitors of cell proliferation. It would be worthwhile to study the effect of arginine and its different methylol derivatives on malignant cell proliferation in tissue cultures and animal experiments.

Acknowledgement

We are indebted to Mr. T. MÜLLER of the Department of Analytical Chemistry, Technical University, Budapest, for analysis of the methylol structure.

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DISTINCT EFFECT OF CONTRACTION AND ION TRANSPORT ON NADH FLUORESCENCE AND LACTATE PRODUCTION IN UTERINE SMOOTH MUSCLE

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The present studies were designed to monitor metabolic perturbations following changes in contractile activity and ion transport in rat and rabbit myometrium by simultaneous measurement of NADH fluorescence, lactate production and isometric force. Stimulation (127 mM K^+) and inhibition (Ca^{2+}-deficient solution) of isometric force development induced a decrease/increase of fluorescence intensity corresponding to a more oxidized/reduced state of tissue pyridine nucleotides, respectively. If tension development was abolished by EGTA, fluorescence changes due to altered ion transport could be monitored. Slow progressive stimulation of the sodium pump by Ca^{2++}-deficient solution resulted in an ouabain (10^{-3} M) sensitive monophasic NADH oxidation, which was reversed by inhibition of the pump by isotonic K^+ solution. If, however, stimulation of the pump was rapid and maximal (addition of 30 mM KCl to Na^+-loaded tissues) the fluorescence response was triphasic and ouabain sensitive: it consisted of an initial NAD reduction followed by a transient NADH oxidation and a second slow NAD reduction. These fluorescence changes are interpreted in terms of separate redox changes in the cytoplasm and mitochondria. The ouabain sensitive stimulation or inhibition of Na/K transport was always accompanied by parallel changes in uterine lactate production irrespective of the actual contractile state of the myometrium. The present tresults show that 1 fluorescence technique can be applied to monitor metabolic perturbations in the uterus evoked by changes in contractility or ion transport; and 2 aerobic glycolysis and Na/K transport are tightly coupled in the myometrium.

Ion transport and contractile activity are dependent on metabolic energy derived from both oxidative and glycolytic ATP productions in the rat myometrium [17, 24]. Previous observations suggested that there was a difference between the sources providing ATP for ion transport and contraction in smooth muscle, but the data are controversial. BUEDING et al. [2] argued that glycolytically generated ATP is more readily available to the contractile mechanism in the guinea pig taenia coli. In contrast, GLÜCK and PAUL [12] suggested that in porcine carotid artery aerobic glycolysis is coupled to Na/K transport rather than to overall cellular energy demands. More recently, PAUL et al. [22] presented evidence that in the porcine coronary artery oxygen consumption bears little relation to Na/K transport even though it is strongly associated with increases in isometric force, and that aerobic glycolysis and Na/K transport are tightly coupled in this tissue. Studying the

effect of ionic environment on O_2 uptake and lactate production in the rat myometrium, KROEGER [17] found that the activation of the ion pump and contractile mechanisms caused strictly parallel changes in the rate of oxidative and glycolytic processes. Thus a distinction between various energy sources providing ATP for ion transport and contraction in the myometrium remained to be elucidated.

In vivo surface fluorometry proved to be a valuable tool for nondestructive kinetic readout of cytoplasmic and mitochondrial redox changes following various metabolic perturbations in skeletal [6, 13, 16, 28] and cardiac muscles [9, 25, 29], but there are no reports available on the application of this technique in smooth muscle.

The present experiments were carried out on the isolated rat and rabbit uteri and metabolic perturbations following changes of ion transport and contractions were monitored by tissue NADH fluorescence and simultaneously by lactate production. Experimental conditions were chosen which allowed to study metabolic consequences of ion transport independently of contractions. The present study provided evidences that aerobic glycolysis and Na/K transport are tightly coupled in the myometrium and that changes in fluorescence are preferentially associated with contractile energy demands. Cellular redox changes associated with ion transport can be detected only in the absence of parallel changes in isometric force development.

Materials and Methods

Preparation of tissue

Twenty-day pregnant rats of the Wistar strain (n = 35) and 25-day pregnant white New Zealand rabbits (n = 7) were killed by a blow on the head, their uterine horns were removed, cut longitudinally along the mesometrial border and the placentae and fetuses were carefully removed. Longitudinal uterine strips (25 mm long and 15 mm wide) were excised and incubated either in normal Krebs solution for 1 hour (fresh tissues) or in K^+-free Krebs solution at 4 °C for 18 to 24 hours (Na^+-rich tissues) [24].

Isometric force recording

The tissues were mounted isometrically in a special water-jacketed and thermostated (37±0.5 °C) muscle chamber which allowed parallel measurement of isometric force, NADH fluorescence and lactate production (see below). The longitudinal uterine strips were fixed on a U-shape plexy frame by 8 pins. The free upper edge of the muscle was connected to a force transducer *via* a platinum hook and chain. The frame was fixed to the bottom of the chamber. Spontaneous and evoked contractions were recorded by a Grass FTO3C force displacement transducer connected to an amplifier and an Esterline Angus rectiliner. A resting axial tension of 1.0 to 1.5 g was applied to all uterine strips.

Fluorometric measurements

Tissue NADH fluorescence measurements were made using a fluorometer basically similar to that introduced by CHANCE et al. [7]. Excitation light was generated by a 100 W air-cooled DC mercury arc lamp (OSRAM HBO 100 W) and the 366 nm ultraviolet excitation wave length was filtered by a Corning glass filter (No. 5874). The excitation light was guided on the muscle surface by means of one branch of a Schott trifurcated quartz light pipe, which was placed into the muscle chamber through a hole in the wall 1 mm from the muscle surface.

The lamp current was electrically stabilized and optical feedback control was achieved by a second quartz lightpipe and a vacuum photodiode (Hamamatsu, R 330). The stability of the excitation light was better than 0.1% at 366 nm. The slow DC drift of the lamp was less than 0.2% per hour. Fluorescence (F) and reflected (R) light were guided by two further branches of the light pipe to end-window photomultipliers (EMI 4524 B) through an interference (F) (450 nm) and a second Corning glass filter (R) (366 nm), respectively. The DC outputs of the phototubes were amplified and recorded by a two-channel Esterline Angus rectiliner. The optical difference signal (F−R = corrected fluorescence (CF)) was produced from F and R signals after filtration and amplification by an electronic differential circuit. The correction factor c was 1. Although theoretically this simple correction does not allow a total compensation of all "tissue density" and "movement" artifacts originating from the special disadvantageous viscoelastic properties of the uterine smooth muscle, the CF signal represented metabolic changes, since the fluorescence responses were either more prolonged and/or had a different time course than the isometric force response.

Since this optical technique required total darkness, the muscle chamber was placed into a well insulated black box (60×30×30 cm) provided by various connections in the wall, which allowed gas supply, solution exchange. drug administration and bath sampling during the experiments.

The fluorescence intensity obtained under basal incubation conditions was deliberately chosen as the 100% level, and changes were expressed in per cents of this value.

Lactate production measurement

Tissue lactate release was measured in aliquots of total bathing solution (20 ml). Samples (1.5 ml) were periodically withdrawn from the bath and immediately frozen (−22 °C) until assayed for lactic acid by the enzymatic method described by HOHORST [15]. The volume withdrawn was immediately replaced. The dilution effect resulting from this procedure (about 7.8%) and the mixing of dead volume in the polyethylene sampling cannula (about 65 μl) were corrected as described by PETERSON and PAUL [23]. The contribution of bacterial contamination to lactate production was minimized by bacteriostasis. Bathing solution lactate concentration was measured in total lactate production (*i.e.* lactate in tissue plus bath) since this allowed serial measurements from a single muscle in various conditions, and changes of lactate release qualitatively reflect changes in total lactate production [19, 23]. Preliminary experiments revealed that following an induced change in the lactate production rate, the rate of appearance of lactate externally was constant in less than 15 min under the given experimental conditions. Lactate production rate was always characterized by steady state values and expressed as μmoles · gram wet weight^{-1} · minute^{-1}. Control measurements indicated that background lactate production from all sources was less than 3% of tissue rates.

Solutions

Composition of the normal physiological Krebs—Ringer bicarbonate buffer (KRB) in millimoles per liter was as follows: NaCl 120, $NaHCO_3$ 24, KCl 4.6, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5, D-glucose 5.5, and insulin 10 mU/ml. Isotonic (127 mM) KCl solution (K^+ — KRB) was prepared by replacing Na^+ for K^+ on equimolar basis. K^+-deficient solution was prepared by ommiting KCl and replacing KH_2PO_4 for NaH_2PO_4. Ca^{2+}-deficient solution was made by ommitting $CaCl_2$ and adding EGTA (0.5 mM) to the solution. All solutions were equilibrated with a gas mixture of 95% O_2 + 5% CO_2 or 95% N_2 + 5% CO_2 to give a pH of 7.4.

Chemicals

The following reagents were used in the experiments: EGTA (ethyleneglycol-*bis*) β-amino ethyl ether (-N,N'-tetraacetic acid) (Sigma Chemicals Co.), ouabain (Sigma Chemicals Co.), lactic dehydrogenase, hydrazine buffer and nicotinamide adenine dinucleotide (NAD) (all from Boehringer Mannheim).

Statistical analysis

The mean and standard error of the mean ($\bar{x}\pm$SE) were calculated and the results were expressed in these terms unless otherwise noted. The statistical difference between samples was estimated by the paired and unpaired Student t test, and was regarded as significant if $p \leq 0.05$.

Results

Incubation of rat uterine strips in Ca^{2+}-deficient solution caused a progressive decline of the intensive spontaneous contractile activity which finally disappeared within 10 min (Fig. 1). The cessation of isometric force development was accompanied by a considerable increase of fluorescence intensity (+14%) and lactate production rate (from 0.26 to 0.48 μmol · g^{-1} · min^{-1}) (Fig. 1). Replacement of Ca^{2+}-deficient solution for isotonic K^+-KRB resulted in an immediate increase of isometric force accompanied by a rapid decrease of fluorescence intensity (—11.5% max) and a significant suppression of lactate production rate to 0.06 μmol · g^{-1} · min^{-1} (Fig. 2).

The redox changes of uterine pyridine nucleotides following alterations of isometric force development agreed well with previous findings in skeletal [16, 28] and cardiac muscles [8]. The aerobic glycolytic rate was reported to be linearly related to isometric force in vascular smooth muscle [21]. In this study, however, lactate production rate increased when contractions were

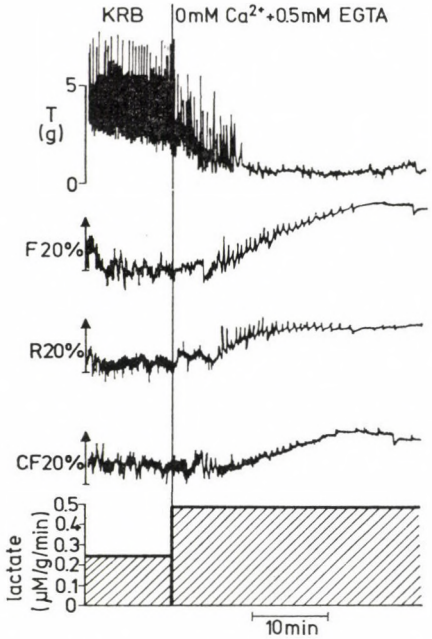

Fig. 1. Effect of Ca^{2+}-deficient Krebs solution on spontaneous isometric force development (T), fluorescence (F), reflectance (R) and corrected fluorescence (CF) (see Methods) and rate of lactate production in uterine strip isolated from a 20-day pregnant rat. The tissue was incubated in normal Krebs solution for 1 h when the bathing solution was replaced by Ca^{2+}-deficient medium containing 0.5 mM EGTA (vertical line) F, R and CF increase are denoted by an upward deflection of the respective traces in this and in all subsequent figures. The dashed area at the bottom represents steady state value of lactate production rate under basal conditions and in Ca^{2+}-deficient solution

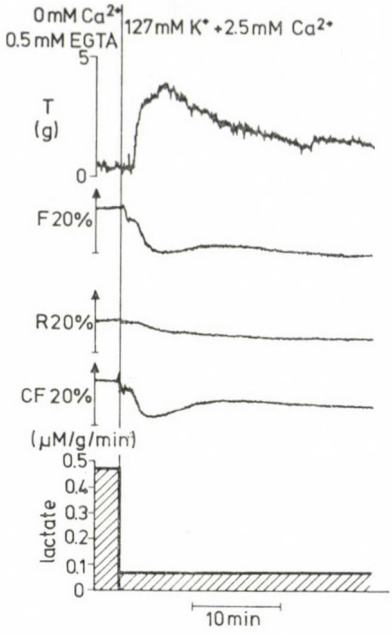

Fig. 2. Record of isometric force (T), fluorescence (F), reflectance (R) corrected fluorescence (CF) and steady state value of lactate production (see Fig. 1) in a rat uterine strip presoaked in Ca^{2+}-deficient solution for 30 min and stimulated by isotonic K^+-Krebs solution at the time marked by the vertical line. Time course of the fluorescence change is very fast after the small stimulus artifact due to exchange of the bathing solution

abolished in Ca^{2+}-deficient solution and it decreased when isometric force was increased by K^+-KRB. These findings suggest that the two ionic conditions used to inhibit and activate uterine contraction might induce changes (of opposite direction) in other energy-requiring cellular process(es) which masked or even reversed the effect of isometric force on aerobic glycolytic rate.

Ca^{2+}-deficient solution was reported to cause membrane depolarization in the rat myometrium due to the increase in membrane ion permeability primarily for Na^+ ions [3, 20]. These permeability changes might have activated the coupled Na/K transport in the present experiments. This assumption was confirmed by the finding that the presence of ouabain (10^{-3} M), a well-known inhibitor of the sodium pump, prevented the stimulation of lactate production by Ca^{2+}-deficient solution (Table I). On the other hand, isotonic K^+ solution was demonstrated to inhibit Na/K pump activity in the rat uterus [17] and the significant reduction of lactate production rate may have been its consequence (see Discussion).

To clarify the relations between lactate production and Na/K transport on the one hand, and between NADH fluorescence and the sodium pump on

Table I

Steady state values of isometric force (T), corrected fluorescence changes (ΔCF) and lactate production rate (LPR) in the fresh and Na^+-loaded rat myometrium under various ionic conditions and during anaerobiosis+

Fresh

	Ionic conditions		T(g)	ΔCF (per cent)++	LPR (μmol · g^{-1} · min^{-1})
	K^+ (mM)	Ca^{2+} (mM)			
1.	5.8	2.5	3.2±0.2*	0	0.372±0.028
2.	5.8	0	nil.	+15.6±2.1§§	0.469±0.032§
3.	5.8	0	nil.	+14.5±2.8§§	0.328±0.022
	+ ouabain				
4.	127	2.5	4.9±0.3§	−16.2±1.8§§	0.060±0.038§§
	N_2		0.6±0.2§§	+31.8±2.5§§	1.240±0.065§

Na^+ Loaded

	K^+ (mM)	Ca^{2+} (mM)	T(g)	ΔCF (per cent)	LPR (μmol · g^{-1} · min^{-1})
1.	0	0	0.4±0.2	0	0.266±0.010
2.	30	0	nil.	triphasic**	0.640±0.052§§
3.	30	0	nil.	0	0.295±0.023
	+ ouabain				
4.	127	2.5	4.3±0.3§§	−14.8±2.1§§	0.055±0.024§§
	N_2		nil.	+30.5±2.2§§	1.277±0.072§§

+ Mean±SE of data from experiments of 12 fresh and 12 Na^+-loaded rat uterine strips. With the exception of ouabain treatment (which was studied in separate experiments) the various experimental conditions were used successively in each uterine strip at approximately 30 min intervals

++ Corrected fluorescence intensity during basal incubation conditions (first row in each part of the Table) was chosen as the 100% level and changes were expressed as differences from it in per cents. An increase/decrease of CF intensity is noted by a plus/minus sign, respectively

* Mean amplitude of the spontaneous isometric tension development

** The characteristic triphasic fluorescence response is demonstrated in Figure 4

Statistical differences between basal (first row) and all other experimental conditions: § $p < 0.05$; §§ $p < 0.001$

the other hand, the metabolism of the myometrium was studied under conditions which allowed to alter the ion transport independently of contractile activity.

Ca^{2+}-deficient solution did not alter isometric force development in the 25-day pregnant rabbit myometrium, since in this muscle spontaneous contractions are lacking due to the suppressive action of the progesterone block [9]. The removal of external Ca^{2+} induced an increase in lactate production rate in this tissue as well (from 0.31 to 0.49 μmol · g^{-1} · min^{-1}), but caused a de-

crease of fluorescence intensity (—14.5%) (Fig. 3). If the sodium pump was then inhibited by K^+-KRB, which did not increase the isometric force due to the absence of external Ca^{2+} and the presence of EGTA, metabolic changes were of opposite direction: fluorescence intensity increased and lactate production dropped to a low level (Fig. 3).

Further experimental confirmation of the relationship between ion transport and metabolism was achieved by studying the effect of various ionic conditions in Na^+-loaded tissues. The addition of 30 mM KCl to the K^+- and Ca^{2+}-free Krebs sulotion (in which Na^+-rich rat uterine strips were incubated at 37 °C for 1 hour, produced a significant increase in lactate production rate (from 0.24 to 0.69 $\mu mol \cdot g^{-1} \cdot min^{-1}$), a transient relaxation and a triphasic fluorescence response consisting of an initial NAD reduction followed by a transient NADH oxidation and a second slow NAD reduction (Fig. 4). These changes were most probably due to the stimulation of Na/K transport since rouabain (10^{-3} M) prevented them (Table I).

The existence of the Pasteur effect in this tissue was demonstrated by endering the muscle hypoxic by equilibrating the bath with a gas mixture of

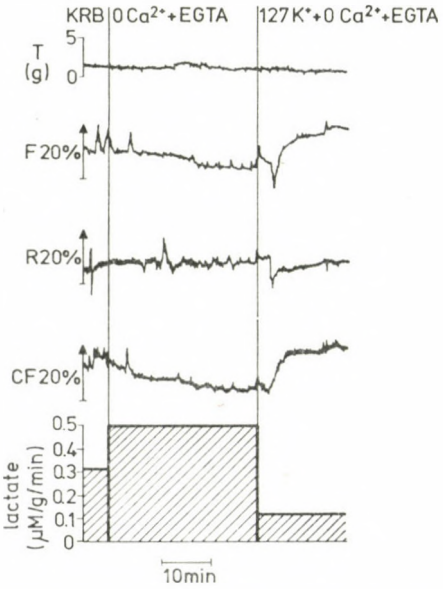

Fig. 3. Record of isometric tension (T), fluorescence (F), reflectance (R), corrected fluorescence (CF) and lactate production (see Fig. 1) in a 25-day pregnant rabbit uterine strip during a sequence of various ionic conditions. Due to the effective progesterone block, spontaneous contractile activity is lacking. The progressive decrease of fluorescence intensity in Ca^{2+}-deficient solution (first vertical line), corresponding to a more oxidized state of pyridine nucleotides, was rapidly reversed on replacing the bathing solution for isotonic K^+-Krebs solution containing no Ca^{2+} and 0.5 mM EGTA (second vertical line). The significant stimulation of lactate production was inhibited by K^+-Krebs solution. Note that all these metabolic changes developed in the absence of isometric force development

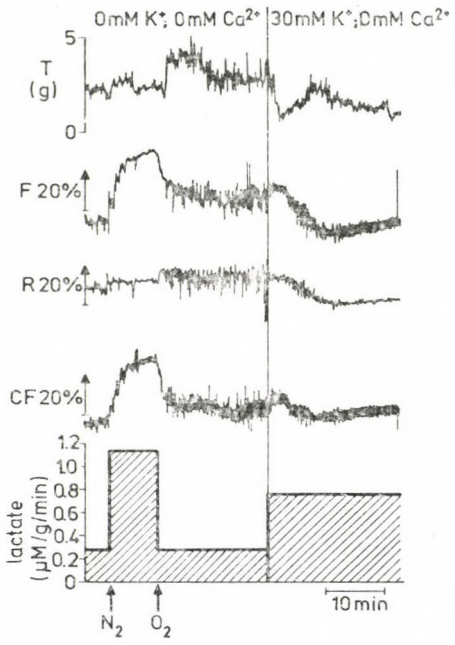

Fig. 4. Record of isometric force (T), fluorescence (F), reflectance (R), corrected fluorescence (CF) and steady state value of lactate production rate (see legend to Fig. 1) in a Na^+-loaded rat uterine strip incubated in K^+-free Krebs solution at 4 °C for 22 h and subsequently in K^+-free and Ca^{2+}-deficient Krebs solution at 37 °C for 1 h. Anaerobiosis (N_2-hypoxia for 10 min) increased fluorescence intensity and significantly stimulated the lactate production rate (4.6-fold increase). Stimulation of the Na/K pump by the addition of 30 mM KCl to the solution (vertical line) caused a transient slight relaxation of the muscle, induced a triphasic fluorescence response and an increase of lactate production rate

95% N_2 + 5% CO_2 for 10 min, which resulted in an aerobic–anaerobic transition of fluorescence and a significant 4.6-fold increase in lactate production (Fig. 4).

Table I summarizes the mean steady state values of isometric force, corrected fluorescence and lactate production rate in various experimental conditions in the fresh and Na^+-loaded rat myometrium. Basal isometric force development and lactate production rate were significantly higher ($p < 0.01$) in the fresh than in the Na^+-rich uteri. Ca^{2+}-deficient solution induced a significant increase of lactate production in the fresh myometrium. This effect was totally prevented by the presence of ouabain (10^{-3} M), while the increase in fluorescence following cessation of spontaneous contractions was not influenced by ouabain. The addition of 30 mM KCl induced a complex fluorescence response (see Fig. 4) and a significantly increased lactate production rate in Na^+-rich tissues, both of which proved to be ouabain-sensitive. Equimolar replacement of Na^+ for K^+ in the Krebs solution evoked an increase

in isometric force and a significant decrease fluorescence intensity and lactate production rate below unstimulated levels in both tissues. N_2-hypoxia caused a significant increase of lactate production in the fresh (3.3-fold) and Na^+-rich tissues (4.8-fold), with a parallel aerobic–anaerobic fluorescence transition of +31.8 and +30.5%, respectively.

Discussion

The present results showed that: 1) pyridine nucleotide fluorescence reflects changes in the cellular redox state associated with altered contractile activity in the myometrium, similar to those observed in skeletal and cardiac muscle; 2) metabolic perturbations following changes of ion transport induce specific complex NADH fluorescence responses only in the absence of parallel changes in isometric force; and 3) stimulation and inhibition of Na/K transport is always accompanied by parallel changes in uterine lactate production irrespective of the direction and extent of changes in contractile activity.

Pyridine nucleotide fluorescence

It is generally accepted that when a tissue is exposed to UV light of 366 nm, the fluorescence emission around 450 nm provides an index of the oxidation–reduction state of the nicotinamide-adenine dinucleotides. NADH is fluorescent and NAD^+ is not, so that a decrease of the fluorescent light intensity corresponds to a more oxidized state of the coenzyme, while an increase corresponds to a more reduced state. Fluorescence techniques have been applied to the measurement of the redox state of pyridine nucleotides in a number of intact tissue preparations. In spite of the fact that skeletal muscle was among the first tissues in which this method was successfully applied [6] and intensive fluorometric studies helped to elucidate the energy metabolism of the heart muscle [9, 25, 29], there are no reports on the application of the method on smooth muscle. The present results demonstrate that in spite of the disadvantageous viscoelastic properties and relative low mitochondrial yield [11] of the uterus, the technique allowed to monitor reproducible and consistent NADH fluorescence changes during a series of metabolic transitions imposed successively on the myometrium.

Contractile activity is the main energy-consuming physiological event in smooth muscle [21]. Metabolic perturbations following activation and inhibition of isometric force development of the myometrium induced marked changes in the myometrium of the rat and rabbit. Sustained force development by high K^+ was accompanied by a rapid change of the pyridine nucleotides toward a more oxidized state (Fig. 2) which is reminiscent of the changes

produced by twitches in excised skeletal [6, 16] and heart muscles [8]. In analogy with studies on respiratory control in isolated mitochondria [5], the conclusion can be reached that these changes were due to stimulation of mitochondrial respiration by ADP and phosphate released during the muscle contraction (State 4 to 3 transition). This suggestion was confirmed by the finding that inhibition of spontaneous contractions by Ca^{2+}-deficient solution induced opposite changes in fluorescence intensity (i.e. NAD reduction) (State 3 to 4 transition) (Fig. 1).

Our findings concerning the changes of fluorescence intensity by ionic conditions which alter the coupled Na/K transport, were more complex. If ionic conditions were able to alter ion transport and isometric force simultaneously, NADH fluorescence changes reflected only alterations in contractile activity (see above). If, however, experimental conditions were chosen which allowed to induce changes in Na/K transport independent or in the absence of contractions, NADH fluorescence responses due to altered Na/K pump activity could be monitored. When the stimulation of ion transport developed slowly (as in the case of the 25-day pregnant rabbit uterus soaked in Ca^{2+}-deficient solution; Fig. 3), the fluorescence intensity decreased progressively, most probably due to the stimulation of mitochondrial respiration by ADP and phosphate released during activation of the transport ATPase (State 4 to 3 transition). This assumption was supported by the rapid reversal of fluorescence changes when the pump activity was inhibited by isotonic K^+ solution. These fluorometric observations correspond to the previous demonstration of increase/decrease of uterine O_2 uptake by stimulation/inhibition of the coupled Na/K transport in the rat myometrium [17].

If, however, stimulation of the sodium pump by 30 mM K^+ in the Na^+-loaded tissue was rapid and maximal [1, 4, 17], the accompanying fluorescence response was triphasic (Fig. 4). These complex changes of the fluorescence are interpreted in terms of separate responses of the pyridine nucleotides in the cytoplasmic and mitochondrial spaces, with the initial increase of fluorescence corresponding to an increase of cytoplasmic NADH and the subsequent fluorescence decrease being due to an oxidation of the mitochondrial component. The third phase may be attributed to interaction(s) between the two spaces either by the operation of a shuttle mechanism *via* the malate–aspartate cycle [30] (the existence of which has not yet been proved in the myometrium), or due to the stimulation of the mitochondrial tricarboxylic cycle producing NADH at a slower rate [13].

The previous [17] and the present findings showing that the stimulation of Na/K transport is always accompanied by an increased lactate production rate in the myometrium (see below) indicate that the flux through the glycolytic pathway is greatly enhanced under these conditions. These results support the rapid accumulation of cytoplasmic reduced pyridine nucleotides by glycer-

aldehyde-3-P-dehydrogenase, similarly as that observed in the heart muscle after epipnephrine-induced stimulation of glycogenolysis [29] or after Ca^{2+} activation of the metabolism [25]. The absence of the initial increase of fluorescence in the rabbit myometrium may be explained by the lack of an initial very rapid and maximal flux through the glycolytic pathway which results in a less of an imbalance between the rate of cytoplasmic NADH production and its removal (i.e. reoxidation by cytoplasmic enzymes or by a shuttle mechanism). The fact that these fluorometric changes were ouabain-sensitive indicate that they were really evoked by changes of the Na/K transport mechanisms. This effect of ouabain proved to be specific, since fluorescence changes due to altered contractile activity were not sensitive to ouabain (Table I).

Further experimental confirmation of the distinction between redox changes in the cytoplasmic and mitochondrial spaces can be expected from future studies using specific metabolic inhibitors and various exogenous substrates other than glucose.

Lactate production

Aerobic glycolysis and consequent lactate production under normoxic conditions have been reported in vascular [22, 23], intestinal [4, 26] and uterine smooth muscle [17]. The present results obtained in the rat and rabbit myometrium support these earlier observations. The cause of aerobic glycolysis is unclear. Tissue hypoxia, insufficient capacity to oxidative phosphorylation and high levels of glucose in experiments *in vitro* were all ruled out as possible explanations of its occurrence in vascular smooth muscle [21] and they can be ruled out on the same basis for the myometrium as well. The concept that aerobic glycolysis indicates a defect in the Pasteur effect [18] can also be ruled out since anaerobiosis was accompanied by a significant severalfold increase of lactate production (see Table I). The possibility that aerobic glycolysis exists due to its firm coupling to ion transport [22], which is of superior importance in the smooth muscle, was supported by the present data on uterine smooth muscle.

The rate of aerobic lactate transport was reported to be related to isometric force in vascular smooth muscle [12, 14, 23]. These findings were confirmed for the uterus as well, since the spontaneously active rat myometrium produced significantly more lactate (0.372 ± 0.028 μmol $\cdot$ $g^{-1} \cdot min^{-1}$) than the inactive 25-day pregnant rabbit uterus (0.278 ± 0.022 μmol $\cdot$ $g^{-1} \cdot$ $\cdot min^{-1}$, $p < 0.05$), under the same incubation conditions.

Exceptions to the concept that aerobic lactate production is dependent on isometric force development were presented in the porcine carotid [12] and coronary artery [22], and in the rat myometrium earlier by KROEGER [17]

and now in the present study (Fig. 2 and Table I). In these cases lactate production did not increase with enhanced isometric force (stimulated by isotonic K^+ solution) but rather remained unchanged or even decreased below the unstimulated level. This differential effect of high K^+ on lactate production may be explained by the suggestion that aerobic glycolysis is coupled to an energy-requiring process which is inhibited by isotonic K^+-Krebs solution. This cellular mechanism may be the coupled Na/K transport which was reported to be inhibited by the total replacement of Na^+ for K^+ in arteries [22], in the intestine [4] and in the myometrium [17].

An alternative explanation suggests a direct inhibition of aerobic glycolysis by high K^+. Glycolytic enzymes could be inhibited by intracellular changes induced by isotonic K^+ medium. There is, however, no direct evidence to support this mechanism.

The findings for Na^+-rich tissues helped to elucidate the relation between lactate production and Na/K transport in the myometrium. In the absence of external K^+, the sodium — potassium pump is inhibited [4, 24] and this led to a significantly lower lactate production rate (Table I). As observed by KROEGER [17] and CASTEELS and WUYTACK [4], this low rate was significantly stimulated by the addition of 30 mM K^+ to the K^+-free solution, which was shown to stimulate maximally Na/K transport in the Na^+-loaded tissues [1]. Since ouabain abolished this effect (Table I), the stimulation of lactate production by 30 mM K^+ was certainly due to the activation of Na/K transport under the present experimental conditions as well. These findings strongly suggest that like in the vascular smooth muscle [22], aerobic glycolysis and Na/K transport are tightly coupled in the rat myometrium.

Rat and rabbit uteri produced more lactate in Ca^{2+}-deficient solution than in the presence of external Ca^{2+} (Figs 1 and 3; Table I). Similar observations were made by KROEGER in the rat myometrium [17], but CASTEELS and WUYTACK [4] found no difference in anaerobic lactate production by the guinea pig taenia coli incubated in the presence or absence of external Ca^{2+}. The difference may be explained by the anaerobic conditions used in the latter study or may be attributed to tissue differences. Another explanation can be related to the fact that removal of external Ca^{2+} may induce several cellular processes, which influence tissue metabolism in different directions. The lack of external Ca^{2+} increases membrane ion permability in the myometrium [3, 20], which may stimulate the sodium pump. On the other hand, the energy-requiring Ca pump is inhibited under these ionic conditions [17]. The third mechanism is related to the fact that the presence of Ca^{2+} is needed to the activation of glycogenolysis by the phosphorylase system [10].

The observed significant increase of lactate production in Ca^{2+}-deficient solution indicates that in spite of the opposite effects of the two mechanisms, stimulation of Na/K transport determined the rate of aerobic glycolysis. The

assumption that Na/K transport was accelerated in Ca^{2+}-deficient solution was supported by the finding that ouabain abolished the lactate production stimulating effect of the Ca^{2+}-deficient solution (Table I).

Although the exact nature of the relationship between aerobic glycolysis and Na/K transport is not known, coupling of the two events *via* membrane-bound glycolytic enzymes would appear to be a plausible model [22, 27].

Finally, the present results indicate that the tight coupling between ion transport and aerobic glycolysis may be a property of smooth muscles in general, and also that smooth muscle can be a good experimental model for a further elucidation of intracellular compartmentalization of energy producing metabolic processes and for the distinction between the sources providing ATP for various energy-requiring cellular mechanisms.

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SEASONAL CYCLE OF GONADAL, THYROID, AND ADRENOCORTICAL FUNCTION IN THE ROOK (*CORVUS FRUGILEGUS*)

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Seasonal function of the gonads, thyroid gland, adrenal cortex have been investigated in male and female rooks, collected in their natural biotop during the characteristic periods of their life cycle. In males, plasma testosterone concentration was found to increase faster than testicular weight at the beginning of couple formation. The thyroxine level increased simultaneously with testosterone concentration. The plasma corticosterone content increased with a phase delay, about one month later. At the end of the reproductive period, testicular weight decreased considerably with a delay of a half month. Before and during prenuptial moult, high triiodothyronine, thyroxine and corticosterone levels were detected. During the resting period in autum and winter the plasma sexual steroid, thyroxine and glycocorticoid concentrations were low. In females, white and small yellow follicles were in an increasing during the nesting-nest-reparing period. During this period the progesterone, an in a smaller degree oestrone and testosterone levels increased. Simultaneously, thyroxine and corticosterone levels increased considerably. During mating, the ovaries were characterized by large yellow follicles. Plasma progesterone and testosterone concentrations decreased, while that of the oestrogens failed to change. The plasma thyroxine and corticosterone contents decreased markedly while the triiodothyronine level increased in a smaller degree. During egg-laying (periovulation phase), the increase in progesterone and oestrone levels was accompanied by an increase in the thyroxine and corticosterone levels. During hatching, the plasma concentration of all the sexual steroids, thyroid hormones and corticosterone showed a decrease. The ovaries regressed rapidly during period before and during postnuptial moult, the quantity of sexual steroids decreased further, while the thyroxine and corticosterone contents increased considerably. In males, the thyreo-adrenocortical synergism, while in females, the progesterone — corticosterone and the thyroxine–corticosterone synergisms appear to play a fundamental role in the regulation of the hormone levels characteristic of the different phases of the seasonal cycle.

There is little information on the seasonal endocrine cycle of birds. Most of them were performed in domesticated or captive birds. Seasonal variations of sexual steroids have been investigated in the drake [1, 4, 7, 9, 10], the domestic hen [8, 31], in male domestic pigeon [11], in male ring dove [6, 15] and in the canary [17]. Among male birds held in captivity, the gonadal cycle of the sparrow [5], the teal [13, 16], the starling [33] and the Agelaius [14] has been described. On the other hand, there are few eco-physiological data on the seasonal cycle of gonads of non-domesticated populations. Seasonal variations of the sexual steroid concentration in the blood plasma has been investigated by WINGFIELD and FARNER in *Zonotrichia* [35] and by PÉCZELY and PETHES [22] in the collared dove.

The seasonal cycle of thyroid function and glycocorticoid production is known only in few species. Seasonal changes of the thyroxine level and its connection to testicular function were investigated in the domestic duck [2, 3, 12] and the teal [13].

Seasonal cycle of thyroxine and triiodothyronine has been described in the sparrow and *Zonotrichia* [32] as well as in the collared dove [25].

Seasonal variations in adrenocortical function have been investigated in the drake [2], in male and female *Passeriformes* [18], in *Zonotrichia* [34] and in the collared dove [25].

In the present work, the function of gonads, thyroids and adrenal cortex of the rook has been analysed in free-living populations. Comparing the seasonal changes ensuing in the plasma hormone concentration, conclusions have been drawn concerning the possible role of hormonal interactions in the regulation of the seasonal functions.

Materials and Methods

In the period 1978—1980, 130 mature and young male and female rooks were collected in their natural biotop in County Pest. The point of time of collection was chosen according to the life cycle of the animals. Accordingly, sexually inactive birds were collected between the middle of October and the middle of March and sexually active rooks between the end of March and the end of April while others were shot after hatching, as well as before and after moult (end of May, July).

The birds were shot between 10 and 13 o'clock. Blood was taken from the shot birds and was stored in cooled (6—8 °C) heparizined centrifuge tubes for 2—4 hours. Wounded birds were not used. The blood was then centrifuged at 3000 rpm, and until analysis, the plasma was stored in polyethylene tubes at −20 °C.

The state of the gonads was controlled by dissection. The weight of the gonads, thyroids and adrenals was determined with an accuracy of 0.2 mg.

Among the sexual steroids, the fraction of progesterone (PROG), testosterone (TEST), oestrone (E_1) and 17 β-oestradiol (E_2) was determined from the diethylether extraction of the plasma, applying Sephadex LH-20 column chromatography [28]. The hormones were determined by the use of the RIA methods described previously [24].

The plasma thyroxine (T_4) and triiodothyronine (T_3) contents were determined with direct RIA methods applied without extraction and separation [26, 27].

The plasma corticosterone content was determined from the dichlormethane extract applying direct RIA method without previous separation.

Intra- and interassay accuracies of the radioimmunological methods applied have been found to be appropriate (2—10% expressed in variation coefficients). For evaluation, variation analysis was applied.

Results

I. *Seasonal cycle of gonadal function*

a) *Female birds*

Weight of ovaries and the plasma sexual steroid concentration were found to be the same in young and sexually inactive birds. In adult rooks collected in the autumn–winter period, the plasma oestrogene concentration proved to be higher.

At the beginning of the reproductive period, couples had formed: within the population this resulted in a loosening of the groups. Courting and agressive behaviour appeared. The groups retired to the nesting area the couples began to repare the nests and to build new ones. During this period, the weight of the ovaries was found to be quadrupled: the white follicles were enlarged and small yellow follicles were also formed. The plasma PROG level was tripled, and the E_1 and E_2 levels also increased to a smaller degree.

Table I

Seasonal cycle of ovarian function

	Young	Sexually inactive	Increasing follicle	Large follicle	Periovulation	Hatching	Feeding of young birds	Postnuptial moulting
PROG			a	b	c	d		
X	695	699	2300	1502	2293	943	970	777
±SD	275	359	880	548	633	428	324	280
n	6	21	8	8	10	11	6	5
TEST			e	f				
X	343	462	757	284	390	264	293	472
±SD	195	209	293	110	238	141	124	136
n	6	21	8	8	10	11	6	5
E_1			g		h	d		i
X	198	286	513	480	727	398	340	186
±SD	59	99	250	168	279	114	120	36
n	6	21	8	8	10	11	6	5
E_2								
X	206	312	294	292	151	106	170	368
±SD	112	110	128	104	79	33	81	181
n	6	21	8	8	10	11	6	5
Ovarian weight			a	j		k		i
X	47	53	237	2860	3774	494	430	292
±SD	18	16	126	517	1359	270	180	85
n	6	21	8	8	10	11	6	5

Abbreviations: PROG — progesterone, TEST — testosterone, E_1 — oestrone, E_2 — 17β — oestradiol

All sexual steroid values are given in pg/ml

a = $p < 0.001$ v.s. sexually inactive
b = $p < 0.05$ v.s. increasing follicle
c = $p < 0.05$ v.s. large follicle
d = $p < 0.01$ v.s. periovulation
e = $p < 0.05$ v.s. sexually inactive
f = $p < 0.01$ v.s. increasing follicle
g = $p < 0.02$ v.s. sexually inactive
h = $p < 0.05$ v.s. large follicle
i = $p < 0.05$ v.s. feeding of young birds
j = $p < 0.001$ v.s. increasing follicle
k = $p < 0.001$ v.s. periovulation

Just before hatching, nuptial flight in pairs was observed and adherence of the couples to the nest was more expressed. Where mating took place, large yellow follicles dominated in the ovaries: their weight was ten times that measured in the previous period. The plasma PROG and TEST levels decreased, but E_1 and E_2 levels failed to show any change. Thus the relative concentration of oestrogens increased as compared to that of PROG.

During egg-laying, the females stayed in the nest throughout the day: they left it to collect food only for short periods in the morning and afternoon. Ovarian weight increased further, the tertian yellow follicles with stalk were characteristic. Eggs were frequently found in the wholly developed oviduct. In this periovulating phase, the increase of plasma PROG and E_1 concentrations was characteristic while E_2 level decreased.

During hatching, the females left the nest only for short periods. The hatching female could frequently be seen to be fed by the male. At the beginning of this period, the characteristic hatching spot developed within one-two days. The weight of the ovaries decreased to one eight: they were characterized by calices and degenerating secondary follicles in the white follicles showed no change in number and size. The plasma concentration of the sexual steroids decreased during hatching: the decrease reached the highest degree in the case of PROG and E_1.

In the first two–three weeks after hatching, the young birds remained in the nest and were fed by both parents. In this period, the hatching spot started to regress, then it disappeared. Ovarian weight did not show any significant change, though calices could not be observed any more. The concentration of sexual steroids did not differ from that detected in the hatching birds. After taking flight, the groups spent the days in larger grasslands and returned to the nests only for the night. At this time, about a month after the hatching, postnuptial moult started. This period was characterized by a further decrease of ovarian weight: the yellow follicles disappeared completely and the number and size of the white follicles decreased. The plasma concentration of PROG and especially that of E_1 decreased, while the TEST and E_2 levels increased. This was the only period, when the oestrogen sprectum was characterized by the domination of E_2 (Table I).

b) *Male birds*

The weight of the testicles of young male birds collected in the autumn–winter period was about half of those of adult male rooks collected in the same period. Differences could also be observed in their spectrum of sexual steroids. In young birds, the $E_1 + E_2$ value was higher than that of TEST. In contrast, the total concentration of oestrogens was the same as the TEST level in adult, sexually inactive birds. The PROG level was somewhat higher in young rooks. As

regards the sexually inactive male and female birds, all the steroids investigated occurred in identical concentration in their plasma and there was no difference between the two sexes.

At the beginning of the reproductive cycle, aggressive behaviour of the males could be observed in the course of mate selection. During this early period, the weight of the testis increased therefold but the vas deferens showed only little change in thickness. A rapid increase of the plasma TEST concentration was characteristic: it was four times higher than in inactive, winter animals.

Table II

Seasonal cycle of testicular function

	Young	Sexually inactive	Courting	Mating	After hatching	Postnuptial moulting
PROG						
X	940	744	840	1061	790	677
±SD	374	349	230	363	174	81
n	6	24	6	6	5	8
TEST			a	b	c	d
X	440	535	2010	4484	1318	699
±SD	230	242	331	2066	415	271
n	6	24	6	6	5	8
E_1						
X	132	239	230	202	243	214
±SD	74	132	143	119	102	95
n	6	24	6	6	5	8
E_2				e	c	d
X	430	258	114	32	178	288
±SD	256	187	36	23	87	98
n	6	24	6	6	5	8
Testis weight (mg)		f	g	h		i
X	20	43	147	3038	2610	165
±SD	6	15	68	1367	875	82
n	6	24	6	6	5	8

Abbreviations as in Table I

All the sexual steroid values given in pg/ml
a = $p < 0.001$ v.s. sexually inactive
b = $p < 0.05$ v.s. courting
c = $p < 0.01$ v.s. mating
d = $p < 0.05$ v.s. after hatching
e = $p < 0.01$ v.s. courting
f = $p < 0.01$ v.s. young
g = $p < 0.02$ v.s. sexually inactive
h = $p < 0.001$ v.s. courting
i = $p < 0.001$ v.s. after hatching

In the days of the nuptial flight and mating, the weight of the testes increased eighty times, to that measured in the resting period while the plasma TEST concentration to double previous level. This means that the phase of rapid increase in the TEST level preceded the increase in testicular weight accompanying the gametogenesis. A decrease of the oestrogen level mainly of E_2 was characteristic of the mating period.

During hatching, the females were fed by the males, and the males went to collect food together with the sexually immature rooks hatched in the previous year. After hatching, the males took part in the feeding of the young birds. In this period, the females hatched in the previous year and now becoming sexually mature, took up with older males. These male birds had presumably no mate. During the hatching period and especially at its end, the plasma TEST concentration decreased, markedly though the weight of the testicles showed little change. In some birds the testes regressed only in a small degree, but the TEST level did not differ from that of inactive birds.

After hatching, the colonies including the young birds went together to collect food. Behavioural patterns characteristic of the reproductive cycle were now followed by social behaviour patterns (indication of food or enemy, alarm, collective movement, collective spending of night, etc.). The weight of the testicles decreased considerably, and the sexual steroid spectrum was characteristic of inactive birds. Moult of the males took place in this period (Table II).

II. *Seasonal cycle of thyroid function*

a) *Female birds*

Thyroid weight as well as T_3 and T_4 levels showed the same values in young and sexually inactive birds. In the period of follicle growth, a decreasing tendency of the plasma T_3 concentration was detected, while the T_4 level increased considerably and thyroid weight remained unchanged.

During the development of large follicles, the T_3 level increased and that of T_4 decreased markedly while the weight of the thyroid gland remained unchanged. During the periovulatory phase, the weight of the thyroid gland began to decrease, with an unchanged T_3, and a markedly increased T_4 level. During hatching, the weight of the thyroid increased due to the increase in its colloid content. At the same time, the T_3 and T_4 levels decreased.

Before and during the moulting period in summer, thyroid weight was characterized by a further decrease, but beside an unchanged T_3 level a remarkable increase in T_4 could be detected.

In three young female birds being in the state of praenuptial moult in spring, a three-times higher T_4 level (4.73 ± 0.36 ng/ml) was found than in

Table III

Seasonal cycle of thyroid function in female rooks

	Young	Sexually inactive	Increasing follicle	Large follicle	Periovulation	Hatching	Feeding of young birds	Postnuptial moulting
T_3				a		b		
X	0.67	0.67	0.45	0.48	0.63	0.36	0.40	0.45
SD	0.16	0.61	0.26	0.29	0.14	0.23	0.27	0.24
n	6	21	8	8	10	11	6	5
T_4			c	d	e	b		f
X	1.53	1.49	4.35	1.35	2.97	1.80	2.05	5.93
±SD	0.94	0.64	1.79	0.40	1.27	0.97	0.99	1.46
n	6	21	8	8	10	11	6	5
Thyroid weight (mg)						g		
X	41	49	46	45	40	61	58	55
±SD	11	10	13	17	18	10	12	13
n	6	21	8	8	10	11	6	5

Abbreviations: T_3 — triiodothyronine, T_4 — thyroxine

Thyroid hormone values are given in mg/ml

a = $p < 0.05$ v.s. increasing follicle
b = $p < 0.05$ v.s. periovulation
c = $p < 0.001$ v.s. sexually inactive
d = $p < 0.001$ v.s. increasing follicle
e = $p < 0.01$ v.s. large follicle
f = $p < 0.001$ v.s. feeding of young birds
g = $p < 0.01$ v.s. periovulation

non-moulting young birds collected in the same period. Plasma T_3 concentration and thyroid weight did not differ from the values in non-moulting birds (Table III).

b) *Male birds*

Thyroid weight as well as plasma T_3 and T_4 concentrations were identical in young and sexually inactive birds.

During the courting and mate-selecting periods, the T_3 level decreased, while that of T_4 exhibited an increasing tendency. Thyroid weight failed to change. In mating male birds with maximum testicular weight, a considerable increase occurred in the T_4 level while T_3 concentration and thyroid weight remained unchanged.

During and after the hatching period, the T_4 level increased further, and the plasma T_3 concentration and thyroid weight also increased.

Similarly as in the females, a three-times higher T_4 level (4.84 ± 0.97 ng/ml) was found in four young male rooks in the state of praenuptial moult as

Table IV

Seasonal cycle of thyroid function in male rooks

	Young	Sexually inactive	Courting	Mating	After hatching	Postnuptial moulting
T_3			a			b
X	0.41	0.69	0.30	0.32	0.58	1.40
±SD	0.21	0.32	0.22	0.18	0.27	0.38
n	6	24	6	6	5	8
T_4			c			
X	1.56	1.83	5.39	4.75	5.70	6.40
±SD	0.73	0.90	1.51	1.93	2.10	0.45
n	6	24	6	6	5	8
Thyroid weight (mg)						d
X	49	55	54	61	74	108
±SD	13	25	20	31	22	42
n	6	24	6	6	5	8

Abbreviations: as in Table II

Thyroid hormone values are given in mg/ml
a = $p < 0.05$ v.s. sexually inactive
b = $p < 0.001$ v.s. after hatching
c = $p < 0.001$ v.s. sexually inactive
d = $p < 0.05$ v.s. after hatching

Table V

Seasonal cycle of adrenocortical function in female rooks

	Young	Sexually inactive	Increasing follicle	Large follicle	Periovulation	Hatching	Feeding of young birds	Postnuptial moulting
CRT			a	b	c		d	e
X	4.88	3.15	9.25	4.00	5.95	4.71	8.10	14.59
±SD	2.77	1.88	3.18	1.25	0.88	2.10	3.80	1.77
n	6	21	8	8	10	11	6	5
Adrenal weight (mg)								
X	46	45	50	55	58	62	64	73
±SD	7	9	8	6	7	6	8	19
n	6	21	8	8	10	11	6	5

Abbreviation: CRT — corticosterone

Corticosterone values are given in ng/ml
a = $p < 0.001$ v.s. sexually inactive
b = $p < 0.001$ v.s. increasing follicle
c = $p < 0.05$ v.s. large follicle
d = $p < 0.01$ v.s. hatching
e = $p < 0.001$ v.s. feeding of young birds

compared to non-moulting birds. The weight of the thyroid gland and plasma T_3 concentration did not, however, differ from those of non-moulting animals (Table IV).

III. *Seasonal cycle of adrenocortical function*

a) *Female birds*

In young and sexually inactive birds, adrenal weight and plasma CRT concentration were essentially similar.

In the growing phase of the follicles, CRT level increased threefold beside an unchanged adrenal weight. Before egg-laying (dominance of large follicles) and during egg-laying (periovulation phase), adrenal weight increased gradually. At the same time, the plasma CRT content decreased considerably: its value was similar in both periods.

In hatching birds, the CRT level was low, and adrenal weight decreased.

After hatching, in the summer period both adrenal weight and plasma CRT concentration increased (Table V).

b) *Male birds*

Adrenal weight and plasma CRT concentration were identical in young and sexually inactive birds.

At the beginning of the reproductive period in the spring, adrenal weight displayed an increasing tendency, while the CRT level did not change. During

Table VI

Seasonal cycle of adrenocortical function in male birds rooks

	Young	Sexually inactive	Courting	Mating	After hatching	Postnuptial moulting
CRT				a		b
X	3.61	2.72	1.94	6.79	6.90	14.25
±SD	1.28	1.20	0.78	2.23	1.80	4.09
n	6	24	6	6	5	8
Adrenal weight (mg)						
X	48	49	56	54	59	62
±SD	10	11	5	12	8	10
n	6	24	6	6	5	8

Abbreviation: as in Table V

Corticosterone values are given in ng/ml

a = $p < 0.001$ v.s. courting

b = $p < 0.001$ v.s. after hatching

the mating period, adrenal weight did not change, but the CRT level increased threefold.

During and after hatching, adrenal weight and CRT level increased further, maximum values were measured in summer in the postnuptial moult (Table VI).

Discussion

Rooks are typical monocyclic birds, hatching once a year. The gonadal cycle of the male birds is similar but more expressed, than that of the smaller *Passeriformes* hatching usually twice [18, 35]. The testes function actively from the third part of March to the middle of May: this is a one month longer period than that of gonadal function of the females. This postponement might be due to the fact, that the young females hatched in the last year and reaching sexual maturity with a delay of about a month, will frequently mate with older males which presumably have remained alone. During sexual maturation, the TEST level increased to eight times, similarly as in the collared dove [22]. It was also characteristic that the increase in weight of the testes and of the TEST level did not run parallel. The increase in testicular function was followed with a certain delay by the increase of their weight. This was presumably due to the effect of TEST upon spermatogenesis and by its stimulatory influence upon sexual behaviour (sexual behaviour affects sexual maturation). The considerable increase in E_2 level appearing together with the high TEST concentration shows that during sexual maturation there are qualitative changes in biosynthesis and or metabolism of the sexual steroids. Thus, the endocrine background of the sexual behavioural patterns appears to be the increased TEST level and the simultaneously decreased E_2 concentration. In the last quarter of the reproductive cycle, a gradual decrease in TEST level had begun: this was followed by a regression of the testes with a delay of a half month the opposite of the process observed in spring.

In male rooks, the seasonal cycle of thyroid function differs from that type described by ASTIER et al. [3] and by ASSENMACHER et al. [2] in the drake, as well as by JALLAGEAS et al. [13] in the male teal, since in the rooks the antagonistic function of the testis and the thyroid cannot be shown at the beginning of the reproductive period. In the rook, the plasma TEST and T_4 content increased simultaneously during the period of mate choosing and mating, though the rate of T_4 increase was slower than that of TEST a similar phenomenon was observed in male collared doves [25]. The simultaneous increase of both hormones in the spring could be explained by the fact that thyroid function is stimulated by the prolongation of day light. This results in an increased T_4 level in doves and rooks, in spite of the inhibitory effect of TEST. This hypothesis appears to be supported by our observation, according

to which the plasma T_4 level of photostimulated, castrated Japanese quails was higher than that of short-day castrated birds [20]. After hatching, and before and during moult, a further increase in the T_4 level could be detected also in rooks, parallel with the marked decrease of TEST concentration. It resulted in the summer maximum of the T_4 level. Thus, the reciprocal changes of TEST and T_4 can be found also at the end of the reproductive cycle of this species.

The low T_4 level of birds collected in winter shows that in rooks the permanently high plasma T_4 concentration fails to play a role in the adaptation to cold.

The plasma CRT content of male rooks did not change in the period of TEST increase, but was considerably enhanced during the further part of the reproductive cycle. Similar changes were found in the collared dove [25]: in this bird the increase in TEST level was accompanied first by a decrease then with an increase of the CRT level. Thus, adrenal function of free-living birds shows that under natural circumstances of life there is no antagonism between the testis and the adrenal cortex [19, 21] evoked by experimental interventions such as castration or TEST administration. The simultaneous increase in the TEST and CRT levels observed in spring might have exogenous and endogenous causes. Adrenocortical secretion is simulated by the prolongation of day light [23] and CRT secretion is increased by thyroid hormones [19, 21, 23]. It may therefore be supposed that the plasma T_4 concentration increasing gradually in spring and the summer might play a role in the simultaneous increase of the glycocorticoid level.

Through central mechanisms, activity of the adrenal cortex could presumably also be stimulated by sexual behaviour and also by the increased aggressivity characteristic of this period.

The CRT maximum detectable after the reproductive cycle in summer can also be found in the collared dove [25] and in several *Passeriformes* [18]. The thyreo-adrenal synergism might have an important role in the appearance of this CRT maximum. Both T_4 and CRT levels were found to be enhanced further by the decrease of the TEST concentration ensuing during regression of the testes, and in this way the characteristic high plasma concentration of both hormones appears when the rooks enter the period preceding their postnuptial moulting.

Comparing the function of the testes, thyroid gland and adrenal cortex of the rook and of the collared dove, one may conclude that the increase in T_4 plasma concentration plays a fundamental role in evoking the postnuptial moult. This is accompanied by a low TEST and a high CRT level in both species. Praenuptial moult of young rooks could also be observed. Moulting birds were collected when the feathers of young age had become brown and those around the bill were lost.

Accordingly, it was the first phase of the entire process, when the feathers of metallic lustre appearing during sexual maturity could not yet be observed. During this moulting period, the high plasma T_4 concentration was accompanied by a low TEST and CRT level. Female rooks in the state of prae-, and postnuptial moult were also characterized by hormonal changes similar to those detected in males. The hormonal background of the post hatching moulting of females seems to be the high T_4 and CRT levels, while in the first part of praenuptial moulting, only T_4 appears to have a fundamental role.

According to data in the literature, T_4 plays a fundamental role in evoking the postnuptial moult in *Passeriformes* [32] and in the domestic duck and teal [3, 13], while CRT has no such a general role. In the drake, the seasonal peak of the CRT level was found in early spring, while that of TEST one month earlier [2]. In both male and female *Zonotrichia*, the CRT maximum recurred in summer, during the second hatching [34]. At the time of postnuptial moulting, the glucocorticoid level was found to be low in drakes and *Zonotrichia*.

The cycle of gonadal activity has characteristic periods also in female rooks. Comparing the growing period of the follicles and the phase of the large follicles, similarly as in the collared dove [22] the small and yellow follicles produce more PROG than the secondary and tertiary follicles. This means that in these birds, a high PROG level appears during maturation, in contrast with the follicular oestrogen phase characteristic of mammals. In the collared dove the oestrogen shows a peak in the period of the large follicles, *i.e.* after the PROG phase. In rooks, their concentration is identical in the growing ovaries and in those with large follicles. In this species, all the sexual steroids showed a maximum during the period of white and yellow follicles. Because of the decrease in PROG level, in rooks the highest value of the oestrogen/progesterone ratio was found in the next period of ovarian development. As to the qualitive distribution of sexual steroids, in the sexually mature female rook the E_1 concentration exceeded that of E_2, in contrast with the Japanese quail [24], the domestic hen [30, 31] and the collared dove [22]. Similar observation has been described in *Zonotrichia* [34]. Further investigations are needed to decide whether E_1 dominancy was characteristic of other species of *Passeriformes*.

As to TEST, its concentration was found to reach a maximum in both the female collared dove [22] and the rook during the growing period of the follicles. This shows that TEST plays an important role in the maturation of the oviduct starting in this period and also, in the aggressive behaviour of the birds.

The second PROG peak appearing in the periovulation phase underlines the role of this steroid in the regulation of ovulation, described in every species of birds so far investigated.

During hatching, the plasma sexual steroids concentration exhibited a sudden decrease and ovaries quickly regressed, presumably in consequence of the antigonadotropic effect of prolactin produced in large quantities.

In the reproductive period, the low T_4 level characteristic of young and sexually inactive female birds showed a significant increase. Similarly as in the collared dove [25], parallel changes in the PROG and T_4 levels could be observed during the phases of this period. In adult Japanese quails [24] laying eggs continuously the plasma T_4 concentration was decreased, thus an ovary thyroid antagonism occurred. On the other hand, when sexual steroids were administered to ovariectomized quails the plasma T_4 concentration decreased following TEST and oestrogen treatment while it increased after PROG administration [29]. Thus, the common inhibitory effect of oestrogens and TEST dominates in the periovulatory phase of quails, while in the collared dove and the rook, in which the sexual steroid level increases much less, a relative dominancy of PROG could be observed. It seems that in female birds, the character of the ovary–thyroid connection depends on the quantitative relations of the sexual steroid spectrum.

Similarly as in the collared dove [25], in rooks the T_4 level decreased during hatching. The phenomenon might be due to the presumably inhibitory effect of prolactin and to the significant decrease of the PROG level.

In female birds, a peak T_4 level could be detected during postnuptial moult in summer. In females, in which the decrease in the sexual steroid level appeared considerably earlier than in males, the increase in T_4 after hatching could hardly be explained by its interaction with the sexual steroids. The change could be evoked rather by the posthatching increase of the plasma CRT concentration. The effect of CRT to increase the T_4 level has been shown in both female and male Japanese quails [21]. The effect of T_4 to stimulate moulting appears to be supported by the fact that the plasma T_4 concentration in young birds at the beginning of praenuptial moulting was found to be threefold that of non-moulting birds of similar age.

During the reproductive period, the plasma CRT concentration showed seasonal changes parallel with the alterations detected in the oestrogen, PROG and T_4 levels. This suggests the stimulatory effect of these hormones found in Japanese quail under laboratory circumstances [23].

During hatching, the CRT level exhibited a decreasing tendency, similar to that described in *Zonotrichia* [34] and the collared dove [25]. The decrease of CRT could be connected white the simultaneous decrease of sexual steroids and thyroid hormones. On the basis of the seasonal maximum of CRT at the time of praenuptial moulting, one may conclude that also in the rook, the thyroid adrenocortical synergism might play an important role in the hormonal regulation of moulting.

The present findings and the few oecophysiological investigations show

that the seasonal cycles in hormone secretion should be interpreted on the basis of their interactions. In addition, they emphasize that the systematical position of birds, and differences in their way of life might result in differences in the realization of hormonal interactions.

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ELECTROPHYSIOLOGICAL INVESTIGATION OF CHEMORECEPTORS OF THE MAXILLARY PALPS OF *LOCUSTA MIGRATORIA MIGRATORIOIDES* R. ET F.

I. GENERAL CHARACTERISTICS OF RECEPTOR RESPONSES EVOKED BY NaCl STIMULATION

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Following stimulation with NaCl of different concentrations, responses from one, two or three receptor cells of the sensillae could be recorded, one of them being always "dominant". The other one or two secondary cells showed a lower frequency and a faster adaptation. The responses of the dominant cells proved to be variable, even to repeated stimulations using the same concentration of the same substance. Increasing the concentration from 0.01 mol/l to 1.0 mol/l, the initial frequency may increase or decrease, however, frequency minimum or maximum may also occur at an intermediate concentration. These findings indicate a less specialized stage of the receptor cells. The individual characteristics of the receptor cells may be surpassed when averaging the responses of sufficiently high number of cells.

On the dome of each maxillary palp about 370 sensillae can be found, the majority (93%) with an open end on them [3]. To each sensilla 6—10 neurones belong, the dendrites of which terminate in the vicinity of the orifice of the sensillae [6]. On the basis of both the morphological and electrophysiological characteristics of the sensillae it has been suggested that their function might be chemoreception [9, 10].

According to HASKELL and SCHOONHOVEN [11], a phase-tonic concentration dependent (0.01—1.0 mol/l) response could be registered following the stimulation of the sensillae with NaCl, saccharose and other substances. In the course of these experiments, the frequency of the responses was found to increase parallel with the increase of the concentration. According to the results of the above authors, one, rarely more cell(s) per sensilla responded to the stimulation, while generally two or three, rarely one or more than three active receptor cells were found by BLANEY [4, 5]. BLANEY's investigations [4, 5] supported the former authors' finding [11], namely, different responses of the sensillae could be registered following identical stimulation. Furthermore, it was also shown by BLANEY [4, 5], that different responses could be obtained from a sensilla even applying the same (10 min) stimulation intervals.

Specific salt, sugar and 'neem' receptors have been distinguished by SCHOONHOVEN [15], and an interaction between them have been established. This latter has been explained by others suggesting adaptation, and the existence of specific receptor cells being sensitive only to one substance has been questioned [4, 5]. According to BLANEY [4], the concentration dependence of the frequency of the sensilla responses is also different, since by increasing the concentration (0.01—0.5 mol/l) increased as well as decreased responses occurred, furthermore at intermediate concentrations responses with frequency maximum also occurred.

Sensillae capable of recognizing different substances or different groups of substances have never been found, for all of the sensillae could respond to many substances in some way [4]. However, 'neutral', 'salt' and 'sugar' sensitive sensillae have been distinguished by "cross-fibre analysis" and so, ". . . the specificity could rather be found to a sensillary than to a neuronal level" [5]. The investigations mentioned above, with one exception [11], have been performed on 3—5. stage larvae and the results appear to be rather contradictory. Therefore, the first aim of our investigations was to clear up the fundamental characteristics (frequency, concentration dependence, stability, etc.) of the excitability of chemoreceptor cells in adult locusts. On the other hand, we attempted to obtain new data concerning the mechanism of food discrimination on the basis of the supposed taste discrimination ability of maxillary palps.

Materials and Methods

Experiments were performed on adult (2—7-days) females of *Locusta migratoria migratorioides* R. et F., from our own breed. The last joint of the maxillary palps were cut and, in order to avoid drying, they were placed into a glass capillary filled with HOYLE's [14] physiological solution containing also 15.84 g/l glucose. A similar preparation, but with more joints was used by HASKELL and SCHOONHOVEN [11].

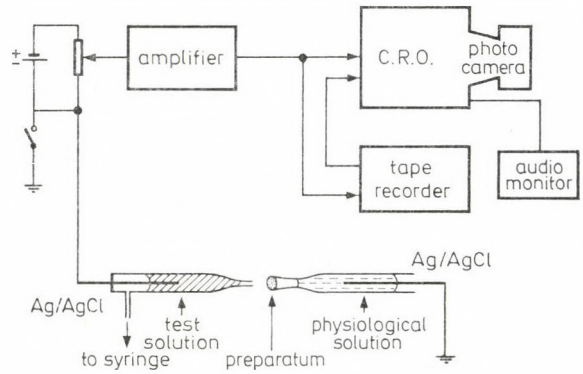

Fig. 1. Schematic representation of the stimulation and registratio n procedure

For electrophysiological recordings the slightly modified technique of HODGSON et al. [13] and BLANEY [4] was applied. To record electrical activity of the sensillae Ag/AgCl electrodes were used extracellularly. The indifferent electrode was grounded, while the different electrode was connected through a compensing circuit to the amplifier. The compensing circuit served for the compensation of the chemical potential difference between the sensilla tip and the capillary containing the stimulating solution and the registrating electrode, as well as to diminish the artefacts arising during touching the sensillae with the electrode (Fig. 1). Compensation was always performed on a neighbouring sensilla.

Glass capillaries with a tip of about 15 μm and containing the stimulating solution and the Ag/AgCl electrode were applied as stimulating/recording electrodes. The electrode was connected to an amplifier used in asymmetrical operation mode and possessing a DC input and variable low-pass filters (1000 Hz) [19]. Electrical activity was recorded with a DISA 51 B 02 oscilloscope and stored by a TEAC R-200c magnetic tape recorder. Stored data were either directly photographed by a COSSOR camera or analyzed with a KFKI NTA 512B 1024-channel analyzer. After measuring the interspike intervals, sequential histograms were made and shown on a msec scale and a frequency scale, or histograms were photographed from the display of the analyzer.

In order to avoid the graduation of the solution in the tip of the stimulating/recording electrode, an injection syringe was connected to the capillary, through which a drop of solution was expressed from the capillary before touching the sensilla with the tip of the capillary. The dropping out of the solution preceded, by 2—5 sec, the mechanical contact. Thus all stimulations were performed with the actual concentration of the solutions. The solutions were diluted from analytical pure stock substances using bi-distilled water.

Results

The first investigation was performed to determine the number of receptor cells of the sensillae responding to the stimulation. Following mechanical stimulation, 70% of the sensillae responding also to chemical stimuli were found to be active. This response could be evoked by the displacement of the tip of the recording electrode, causing bending of the sensilla.

Stimulating the sensillae with NaCl solution, a phase-tonic response could be recorded, in which the activity of maximum 3 receptor cells could be distinguished on the basis of the size of amplitudes, the regularity of interspike intervals and the degree of adaptation (Figs 2, 3). Only one neurone was found to be active in 47% of the sensillae analyzed, while 40% contained two and 13% three active receptor cells. However, the majority of the obtained responses originated always from one receptor cell ("dominant cell"). Thus, the number of potentials originating from the other cell (1—2 secondary cells) were always few and their frequency was found to be considerably lower. Within the response of a sensilla the activity of the secondary cells ceased generally soon (within 1—5 sec). Occasionally, their activity could also be recorded even 10—30 sec after the beginning of the stimulation, though with a very low frequency (0.2—0.5 cps). Eventually, the activity of the secondary cells appeared only 10—30 sec after starting the stimulation. In this case, the frequency of the spikes was found to be higher, reaching 5—10 cps, and could surpass that of the "dominant" cells fairly adapted at this time. The spike series of the dominant cells proved to be more regular than that of the secondary cells.

The adaptation of the active receptor cells during stimulation of the individual sensillae has also been investigated. An example for this is shown in Fig. 4, where the response of three cells is recorded from one sensilla. When applying stimuli at every 10 sec, only one secondary cell was found to be active, in addition to the dominant cell, following the second stimulus (Fig. 4b).

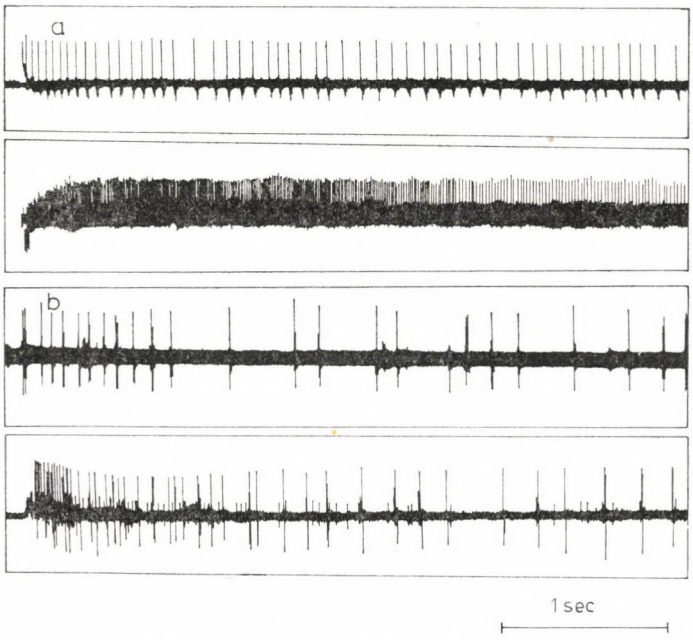

Fig. 2. Responses of sensillae containing one (a) or two (b) receptor cells

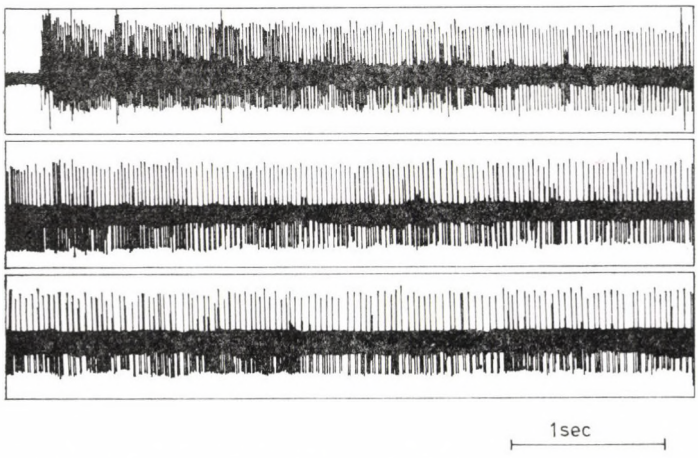

Fig. 3. Response of three receptor cells. The adaptation of potentials with high and small amplitudes is faster. Continuous recording

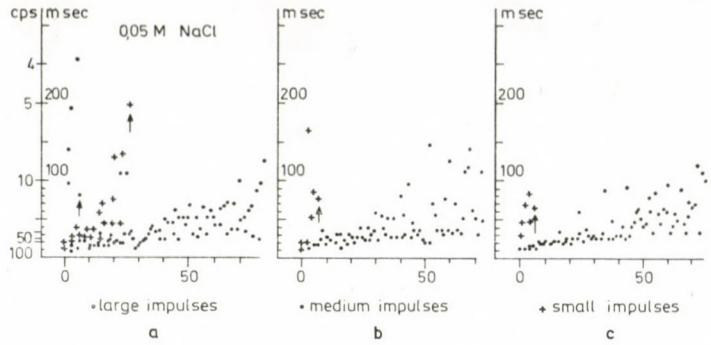

Fig. 4. Adaptation of three receptor cells of a sensilla during repeated stimulation with 0.5 mol/l NaCl for 10 sec. Responses given to the first (a), second (b) and third (c) stimulation are shown. Last potentials of the receptor cells are indicated by arrows. Abscissa: sequence of interspike intervals

This secondary cell exhibited a faster adaptation than after the first stimulation. The number of potentials present in its response decreased further on the third stimulation (Fig. 4c), and it failed to give any response following the fifth stimulation. The responses failed to reappear after a long, even an hour long, resting period. When applying the same concentration of the tested substance during the rest of the experiment, there was not a single case in which the number of receptor cells would have increased compared to that observed at the first stimulus.

Analyzing the response of many sensillae, medium amplitudes proved to be the most stable among the three categories of amplitudes. It did not cease either during an experiment lasting for hours (4—8 hr), if the stimuli were sufficiently rarely repeated.

Determination of the number of active receptor cells during stimulation of the sensillae with NaCl of different concentrations revealed that decrease of these receptor cells occurred in only some of the sensillae. This was probably due to the adaptation to the successive stimuli rather, than being the result of the concentration change. In contrast, Fig. 5 shows a sequential histogram in which only one receptor cell was activated at a NaCl concentration range of 0.01—0.05 mol/l, while at 0.1 mol/l two, and at 0.5 and 1.0 mol/l three cells. Thus, the number of the receptor cells on this sensilla increased with increasing concentration. However, the cells appearing later in the response exhibited a considerably lower frequency and faster adaptation, comparing to the dominant cell being active at all the concentrations applied. In another case most of the cells gave a response at an intermediary concentration. Apart from the above examples, several other variations have also been observed, nevertheless, a concentration dependence in the number of active cells could not be established. When increasing the concentration, the amplitude increased

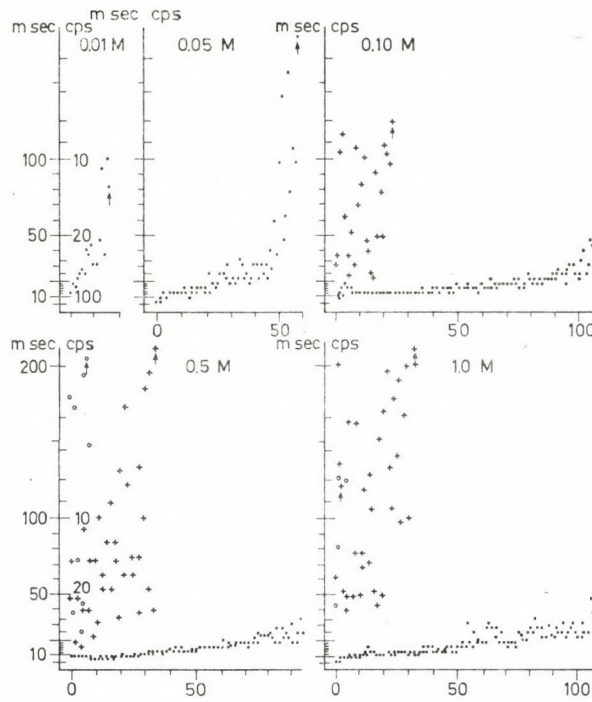

Fig. 5. Changes in the activity of the receptor cells of a sensilla stimulated with NaCl of different concentrations. Symbols as in Fig. 4

in all cells and this was found also to be valid for the potentials of responses evoked by mechanical stimulation.

The above results indicate that it was not possible to find any connection between the number of receptor cells responding to the stimulation of the sensillae and the concentration of NaCl applied for stimulation. Therefore, the responses of the dominant cells were only analyzed in the subsequent experiments.

(1) *Adaptation of the responses*

A typical example of the phase-tonic response of the dominant cells is shown in Fig. 6, following stimulation with 0.01 mol/l NaCl. The initial frequency of the phasic part is 75 cps, which quickly decreased to about 35 cps. Thereafter the adaptation was slower and regular, and after a stimulation with 22 sec the frequency of the response was about 20 cps. Simultaneously with the increase of the duration of interspike intervals, the deviation of the durations also increased. The initial frequency of the receptor cells was found to be 17—160 cps, and the shortest period during which the response was maintained

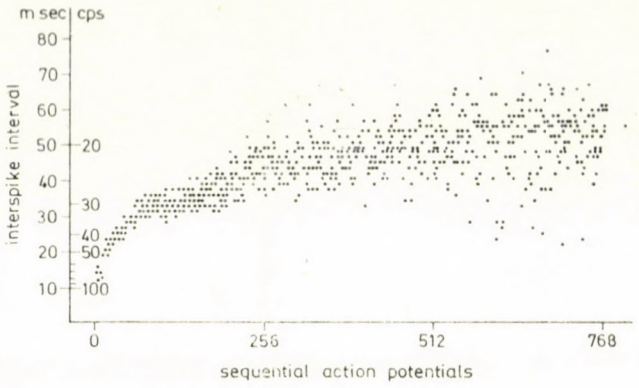

Fig. 6. Typical phase-tonic response of a dominant cell given to stimulation with 0.01 mol/l NaCl. Total registration time: 22 min

attained 1.5 sec. The short responses exhibited a low initial frequency which could not, however, been interpreted as the initial frequency being by all means responsible for the fast adaptation. The duration of the responses was not analyzed in all receptor cells, however, in some cases the response failed to appear even after continuous stimulation for 5 min.

(2) *Stability of the responses*

The inter-stimuli interval dependence of the dominant cell responses evoked by repeated stimulations has been investigated. An example is shown in Fig. 7, where the use of 10 sec intervals during the stimulation with 0.1 mol/l NaCl, resulted in a slight increase of the initial frequency of the response, while its adaptation decreased. At the next stimulation 20 min later, the

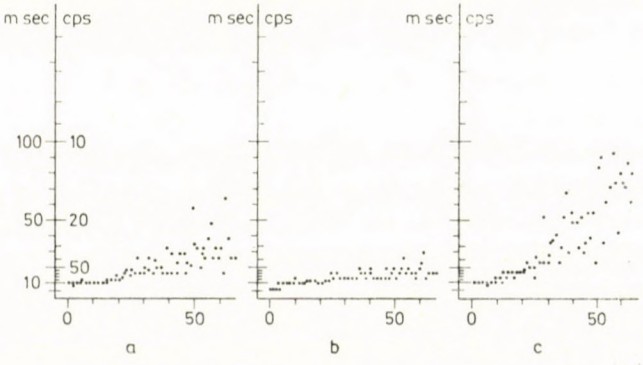

Fig. 7. Responses of a dominant receptor cell to repeated stimulation with 0.1 mol/l NaCl; a) first stimulation, b) second stimulation after 10 sec, c) third stimulation after 20 min. Abscissa: sequence of interspike intervals

Table I

Change of initial frequency of the dominant receptor cells on stimulation with NaCl, using variable interstimulus intervals

Concentration of NaCl solution, mol/l	Interstimulus interval, sec	Change of initial frequency		
		no change, %	increased, %	decreased, %
0.01	5	83	17	0
0.05	5	25	0	75
	10	0	72	28
	20	67	33	0
	30	0	66	34
0.1	5	11	44	45
	10	17	66	17
0.5	5	33	26	41
	10	17	0	83
1.0	5	0	80	20

initial frequency was almost identical with that of the control and its adaptation increased. However, such cases also occurred when the frequency of the response increased following stimulation 30 or 60 min later.

Table I shows the changes of the initial frequency of the responses of different sensillae following stimulation with NaCl of different concentrations and using different stimulation intervals. On the basis of the data presented here it is obvious that the tendency of the changes is independent of the stimulation intervals applied.

(3) *Concentration dependence of the responses*

The concentration dependence of the frequency of responses of the dominant cells was investigated at NaCl concentrations of 0.01 0.05, 0.1, 0.5 and 1.0 mol/l. The threshold concentration of NaCl was found to be below 0.01 mol/l in the majority of the receptor cells (90%), and most of them (70%) responded also to the concentration of 1.0 mol/l. The sequential histogram of the interspike intervals of a cell obtained at different NaCl concentrations is shown in Fig. 8. A more marked response with higher frequency was evident at lower concentrations. At a concentration of 1 mol/l, not only the initial frequency appeared to be smaller but also the frequency decrease in the response was more expressed.

Furthermore, the initial frequency of the responses of 19 receptor cells (calculated on the basis of the time interval between the two first potentials)

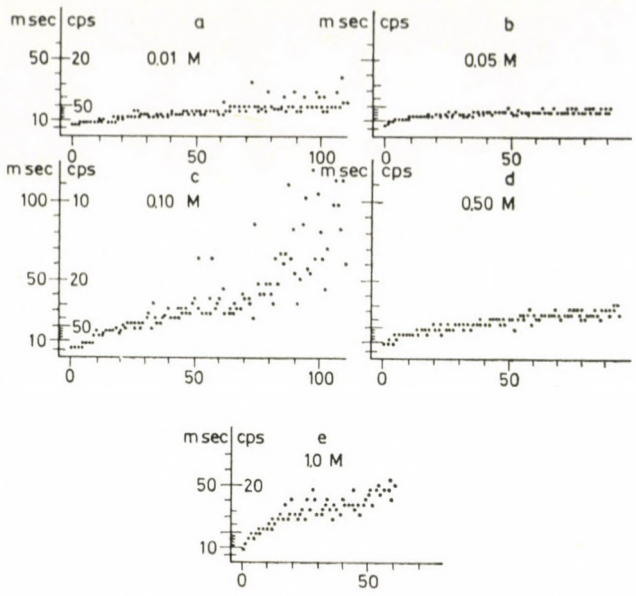

Fig. 8. Responses of decreasing frequency of a dominant cell given to the increasing concentration of NaCl. Abscissa: sequency of interspike intervals

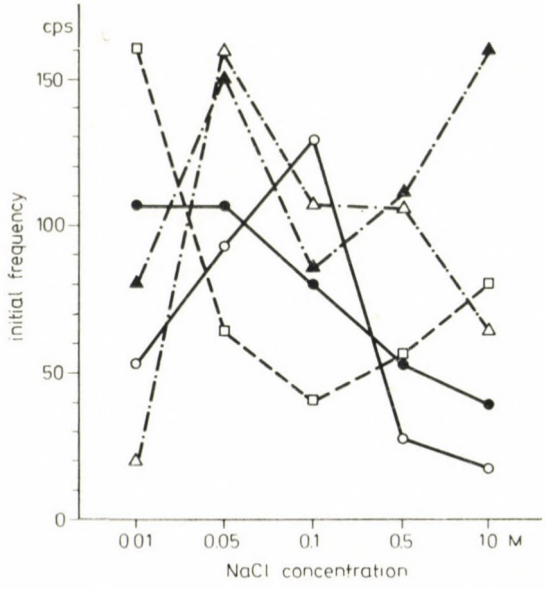

Fig. 9. Initial frequency of the responses of 5 receptor cells stimulated with NaCl of different concentrations

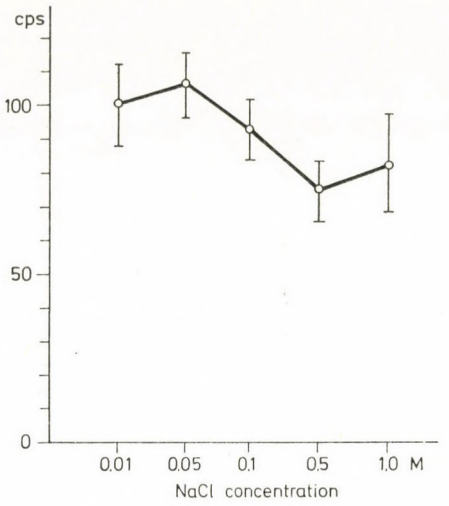

Fig. 10. Average of the initial frequency of the responses arranged according to the applied NaCl concentrations. Vertical bars: standard error of mean

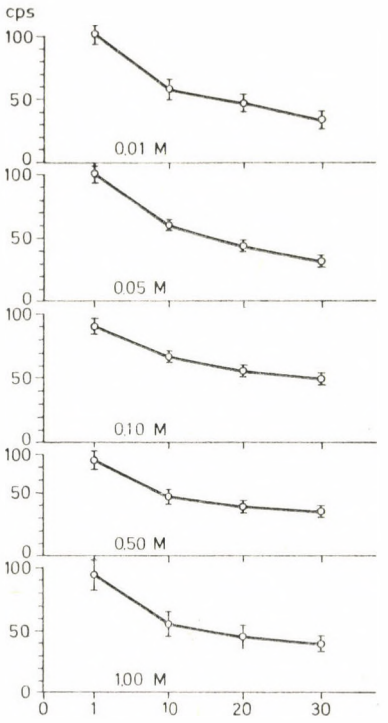

Fig. 11. Frequency decrease of receptor cell responses up to the 30th spike when using different NaCl concentrations. The individual values represent the means of the responses of the same 19 cells. Vertical bars: standard error of mean

was analyzed when applying NaCl solutions of 0.01 mol/l and 1.0 mol/l concentrations. The lowest initial frequency was found to be about 20 cps (between 17 and 23 cps), and it could be observed with all concentrations tested. The highest initial frequency was uniformly 160 cps, with the exception of the concentration of 0.5 mol/l, at which this value was 106 cps. The concentration dependence of the responses of individual receptor cells exhibited marked heterogeneity (Fig. 9). Four basic patterns of responses could, however, been distinguished. With increasing concentration, the initial frequency increased in 15% of the cells, while in 30% it decreased; in a further 30% a frequency maximum and in 25% a frequency minimum was found at an intermediary concentration. As regards the concentrations, 26% and 26% of the cells investigated showed an initial frequency maximum at 0.01 mol/l and 0.05 mol/l, respectively, while in 20% of the cells this value was found to be at 0.1 mol/l, and in 14% and 14% at 0.5 and 1.0 mol/l, respectively.

The mean value of the initial frequency of the responses recorded from the above receptor cells is shown in Fig. 10, following stimulation with different NaCl concentrations between 0.01 and 1.0 mol/l. There was no significant difference between the means of initial frequencies of the responses obtained with varying concentrations.

As it has already been mentioned, the receptor cell responses evoked by chemical stimulation exhibited a phase-tonic character. The frequency changes of the responses of the above cells were analyzed on the basis of the first 30 interspike intervals. The mean frequency values calculated at the 1st, 10th, 20th and 30th interspike intervals are shown in the Fig. 11, following the application of different NaCl concentrations. All the concentrations tested the most marked decrease of frequency was evident during the first 10 potentials of the responses.

Discussion

Since there are two tracheae sacs in the dome of the palps preparation used by us it is rather difficult to make an electric contact between the physiological solution of the indifferent electrode and the solution of the different electrode [11]. Depending on the feed, the terminal pore of the sensilla is opened or closed [2], and this process is regulated through the suboesophageal ganglion by the hormone of the corpora cardiaca [1]. The advantage of the present isolated preparation in the elimination of this regulation.

On the dome of the maxillary palps of the locust most of the sensillae are connected to 6 or 10 neurones, 6 neurones being more general. The dendrite of one of the neurones contains many microtubules and shows branching pattern in the region of the receptor-lymphatic cavity. In the locust, it has not yet been established whether this dendrite containing the tubules termi-

nates at the basis of the sensilla or runs further up to the tip [6]. Such a morphological appearance is usually associated with mechanoreceptor functions [17], and such dendrites terminate at the basis of the sensilla.

When stimulating the sensillae of the maxillary palps, the response of maximum 4 neurones could be distinguished from that of 6—10 ones. The response of one of these 4 neurones could be evoked by mechanical stimulation and in each case it showed the highest amplitude and was detectable in the majority (70%) of the sensillae responding to chemical stimulation. Mechanoresponses could be elicited from all dome receptors by HASKELL and SCHOONHOVEN [11], while only from 47% of the sensillae by BLANEY [4].

It is generally accepted that impulses of different or constant amplitudes originate from different neurones, though it does not mean at all that impulses of the same size are derived from the same neurones [7, 8, 12].

Owing to the small amplitude of the receptor potentials the signal/noise ratio remains below the level encountered in extracellular recordings. Thus a difference of 15—20% could also be observed between the potentials originating from one cell. In such case, the number of active cells could rather precisely be determined by considering the amplitude and interspike interval values, provided that one or more active cells possess sufficiently regular frequency. By this method the response of maximum 3 cells could simultaneously be distinguished during stimulation with NaCl and this attained 13% of the sensillae investigated. In 40% of the sensillae two receptor cells and in 47% only a single receptor cell responded to the stimulation. With NaCl stimulation the response of only 1 neurone/sensilla/adult insect and isolated palp preparation was recorded by HASKELL and SCHOONHOVEN [11], while generally the response of 2 or 3, rarely 1 or more than 3 neurones (larval stage and intact animals) was observed by BLANEY [4, 5]. Since these investigations were performed on locusts being in different developmental stage and on different preparations, any attempt to explain the inconsistency of these findings would be speculative. It seems also impossible to provide satisfactory explanation for the finding that maximum 3 of the 6 receptor cells present in the sensillae responded to the stimulation, since all of them appear morphologically to be determined for chemoreception [6]. The existence of specific chemoreceptor cells not responding to stimulation with NaCl can be excluded on the basis of the present findings and of data of others [18]. It has, however, been shown that the number of receptor cells responding during repeated stimulation of a sensilla decreased from 3 or 2 to 1, and at identical stimuli we never observed the activation of a new cell being earlier silent. Nevertheless, the number of active receptor cells could be altered during the stimulation of a sensilla with NaCl solutions of different concentrations. According to BLANEY [4], the individual sensillae exhibit similar distribution of amplitude categories at each concentration, though a considerable variability in the amplitude

values themselves has been found. It is not clear from his paper, whether the cells belonging to the sensilla showed any difference in adaptation. We also failed to observe a correlation between the applied concentration and the number of active cells. Remarkably, in the present study the spike number and the frequency of the secondary cells were always found to be considerably smaller, than that of the dominant cell. It is conceivable that this phenomenon was due to the receptor cells being alternatively active. Thus, while one cell is active, *i.e.* it is in the dominant or most sensitive state, the others are silent or unresponsive, eventually they only show a partial response with a faster adaptation and lower frequency, characteristic for the so-called secondary cells. Since we failed to find specific receptor cells responding only to a single specific substance when testing a wide range of substances [18], it seems unlikely that the application of an inadequate stimulus would have been the reason for the silence of the cells.

Similarly to larvae, the chemoreceptors of the maxillary palps of adult locusts would also respond with different frequency to identical sequential stimuli [4, 5, 16]. This is true also when stable and sufficiently high stimulus intervals are applied. Thus this pattern does not seem to be due to the adaptation or facilitation of the receptor cells, since the frequency changes of the cells appear to be accidental without any tendency for regularity.

Consequently, a close correlation between the initial frequency of the dominant cells and the concentration of the applied substance cannot be expected. As we have shown, with increasing concentration 15% of the cells exhibited an increasing, and 30% a decreasing initial frequency, while at 30% a frequency maximum and at 25% a frequency minimum occurred when one of the intermediate concentrations was applied. In the two latter groups it frequently occurred that they responded with identical initial frequency to two concentrations being markedly different. In adult locusts, so far only the increasing response to rising concentration has so far been described [11], while the response pattern characterized by a frequency minimum at an intermediary concentration is also unknown in larvae [4].

On summation and averaging of the responses of more cells, the variability of the responses of the receptor cells as well as the dependence of their characteristics on the applied concentration were mutually compensated for and marked. These mechanisms might be important in the process of taste discrimination and food selection.

Our findings clearly show that the receptor cells of the dome sensillae of the maxillary palps can be stimulated by chemical substance (NaCl). A given receptor cell is capable of responding at different time intervals and with different frequency to a given concentration of the substance and this response is independent from adaptation or facilitation. Different receptor cells may respond with different frequency to the same concentration and also, with

the same frequency to different concentrations. When increasing the concentration, the frequency may increase or decrease, however, frequency maxima or minima may also occur at intermediate concentrations. In the case of NaCl, the threshold concentration of the majority of the receptor cells was below 0.01 mol/l and the majority of the cells responded even to a concentration as high as 1.0 mol/l. These features indicate that the receptor cells of the dome sensillae are not specialized to chemical substances, and they are sensitive to a wide range of substances.

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EFFECT OF BILE ACIDS ON THE BILIARY EXCRETION OF AMARANTH AND THE RESPIRATION OF LIVER MITOCHONDRIA

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The effects of lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DOCA), cholic acid (CA) and dehydrocholic acid (DHCA) on the biliary excretion of amaranth (AM) in rats and on the respiration of isolated liver mitochondria were investigated. Bile acids diminished the biliary excretion of AM, and *in vitro* they enhanced the state 4 mitochondrial respiration and inhibited DNP- and ADP-stimulated oxygen consumption. The ID_{50} values (μmol/kg i.v.) and I_{50} concentrations ($\times 10^{-5}$ M) of the bile acids for inhibiting AM excretion and ADP-stimulated mitochondrial respiration, respectively, were as follows: for LCA 16 and 3.3, for CDCA 160 and 24, for DOCA 230 and 31, for CA 680 and 105, and for DHCA 700 and 260. LCA, CDCA and DOCA inhibited the biliary excretion of AM uncompetitively.

It is concluded that the toxic effect of LCA, CDCA and DOCA on mitochondria might play a role in their inhibitory action on the biliary transport of AM.

Bile acids can exert both a stimulatory and an inhibitory effect on the biliary excretion of cholephilic organic anions. It appears that cholic acid is involved in the regulation of the biliary excretion of rose bengal, indocyanine green, sulfobromophthalein, bromcresol green and eosine, since their excretion rates are increased by simultaneous administration of taurocholate [10, 31, 32] and decreased by experimental bile acid depletion [13]. The transport of other organic acids, namely sulfobromophthalein glutathione and amaranth into bile, however, seems insensitive to bile acid deficiency [14] and their excretion can not be stimulated by taurocholate. Moreover, high doses of this bile acid depress their biliary excretion [10].

The present investigation was designed to study the inhibitory action of some unconjugated bile acids, namely lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DOCA), cholic acid (CA), and dehydrocholic acid (DHCA) on the biliary excretion of AM. Since bile acids uncouple oxidative phosphorylation in isolated liver mitochondria [26] and biliary excretory rates of cholephils seem to be affected by inhibition of hepatic mitochondrial function [2, 12], it was of interest to determine if any parallelism existed between their effects on mitochondrial and hepatic excretory functions.

AM is a nonmetabolized organic anion [20] excreted mainly in bile [29] at a high maximum excretory rate. It does not have a cholestatic effect or

inhibit hepatic mitochondrial respiration [12]. In order to minimize a possible interaction between endogenous and injected bile acids, bile acid-depleted rats were used in the present experiments.

Materials and Methods

Materials

Lithocholic acid and cholic acid were purchased from Merck Ltd., Darmstadt; chenodeoxycholic acid from Sigma Inc., St. Louis; sodium salt of deoxycholic acid and dehydrocholic acid from Reanal, Budapest; amaranth (Azorubin S) from Searle Co. (Hopkin and Williams), Chadwellheath; cholestyramine from Serva Co., Heidelberg.

Animal experiments

The experiments were performed on female Sprague-Dawley rats weighing 160—200 g. The animals were pretreated with cholestyramine (1.0 g/kg p.o.) as described elsewhere [14]. Four hours after cholestyramine administration the rats were anesthetized with urethane (1.2 g/kg i.p.) and the bile duct was cannulated with polyethylene tubing (PE-10). The body temperature of the rats was maintained at 37 °C by means of a heat lamp. AM was injected into the femoral vein, then 20 min later the bile acids were administered by the same route. LCA was dissolved in propylene glycol, the other bile acids, as sodium salts, in saline. Propylene glycol administered in the highest volume (2.5 ml/kg i.v.) did not influence the biliary excretion of AM. Bile was collected for 20 min after administration of the bile acids. The volume of bile was measured with a graduated pipet. The concentration of AM in the bile was determined spectrophotometrically at 525 nm. The biliary excretion rate of AM was calculated as the product of biliary flow and biliary concentration.

Mitochondrial respiration

Rat liver mitochondria were prepared by the method of HOGEBOOM [17]. The protein concentration of the mitochondrial suspension was determined by the biuret method [9]. Oxygen consumption of the mitochondria was measured with a Clark electrode at 37 °C and pH 7.4 in 1.4 ml of an incubation medium consisting of 80 mM KCl, 20 mM Tris, 1 mM EGTA and 5 mM KH_2PO_4. A mitochondrial suspension (containing 2 mg of protein), 1.5 μmol malate and 6.5 μmol glutamate were added to the reaction mixture (state 4 respiration). The oxygen consumption was stimulated by adding 1.5 μmol ADP or 50 nmol DNP. The respiratory control index of mitochondria was 10—12. The bile acids investigated were added to the incubation mixture 60 sec after addition of glutamate and malate or 30 sec after addition of ADP or DNP. LCA was dissolved in propylene glycol, the other bile acids, as sodium salts, in the incubation medium. Propylene glycol applied in the highest volume (20 μl) did not influence mitochondrial respiration. The rate of oxygen consumption after addition of a bile acid was related to the oxygen consumption measured before delivery of the bile acid.

Statistics

Student's t test was used for statistical analysis. The lines for Lineweaver-Burk plots were determined by linear regression.

Results

Effect of bile acids on the biliary excretion of amaranth

The effect of three different doses of bile acids on the biliary excretion of AM is presented in Table I. All bile acids investigated inhibited the excretion of AM into the bile. The approximate ID_{50} values for the bile acids are as

follows: LCA, 16, CDCA 160, DOCA 230, CA 680 and DHCA 700 μmol/kg i.v. LCA decreased, DOCA did not influence, CDCA, CA and DHCA increased the bile flow. The concentration of AM in the bile was considerably reduced by the administration of all bile acids except LCA.

The mode of inhibition the bile acids exerted on AM transport was different as demonstrated by the Lineweaver-Burk plots (Fig. 1). LCA, CDCA and DOCA inhibited the biliary excretion of AM in an uncompetitive fashion. However, CA and DHCA produced competitive rather than an uncompetitive type of inhibition.

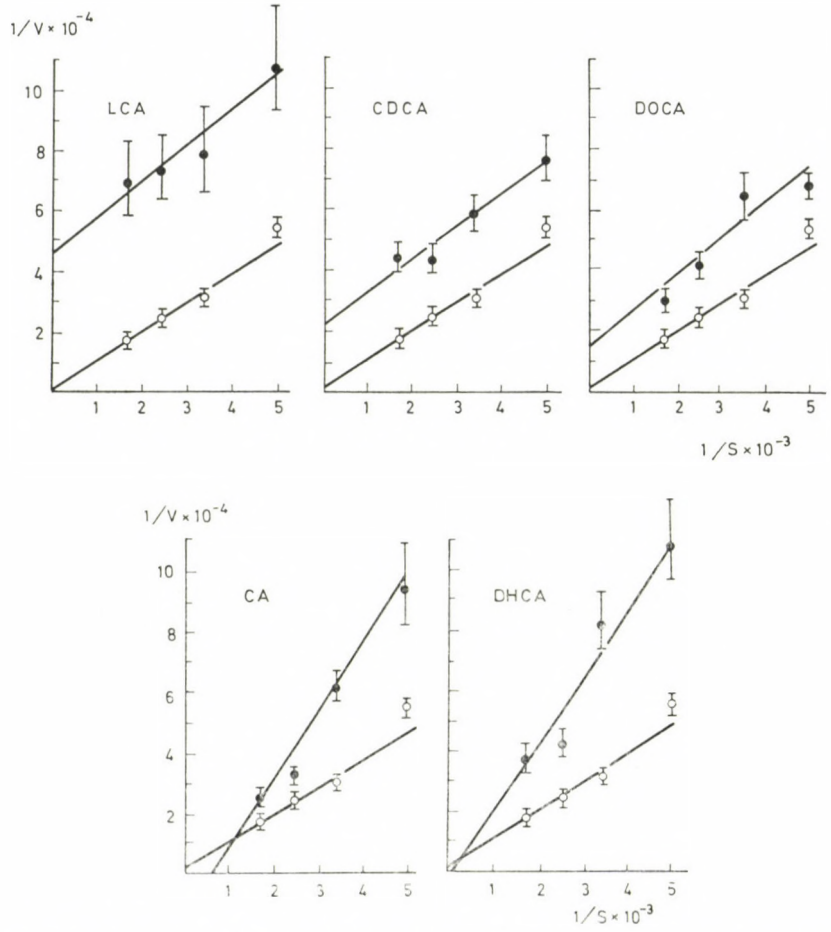

Fig. 1. Inhibition of the biliary excretion of amaranth (AM) by bile acids. Bile acids were injected 20 min after the administration of AM (200, 300, 450, 600 μmol/kg i.v.), and the biliary excretion rate of AM was determined in the subsequent 20 min period. Doses of bile acids in μmol/kg; LCA 20, CDCA and DOCA 200, CA and DHCA 500. V = biliary excretion rate of AM (nmol/kg · min), S = dose of AM (μmol/kg i.v.), ○——○ = the excretion rate of AM without bile acid administration. ●——● = the excretion rate of AM following bile acid administration. Each open or solid circle represents the mean value of 4—6 rats

Effects of bile acids on mitochondrial respiration

The effect of various unconjugated bile acids on respiration of isolated rat liver mitochondria is shown in Table II. The mitochondrial oxygen consumption measured in the presence of glutamate and malate was enhanced by LCA, CDCA, DOCA, and CA and it was not affected by DHCA. The stimulatory effect of the bile acids increased with increasing concentrations, and after having reached a maximum, it decreased. The stimulatory effect increased in the following order: LCA > CDCA > DOCA > CA.

The mitochondrial respiration stimulated by DNP or ADP was inhibited by the bile acids. The approximate I_{50} concentrations of the bile salts for the ADP-stimulated respiration of the mitochondria were as follows: LCA $3.3\times\times10^{-5}$ M CDCA 2.4×10^{-4} M, DOCA 3.1×10^{-4} M CA 1.05×10^{-3} M, DHCA 2.6×10^{-3} M.

Table I

Effect of bile acids on the biliary excretion of amaranth[a]

Bile acids	Dose μmol/kg i.v.	Bile flow μl/kg · min	Concentration of AM in bile μmol/ml	Biliary excretion of AM μmol/kg · min
—	—	67± 3.8	50.7±2.0	3.40±0.189
Lithocholic acid	6.2	66± 3.2	41.2±2.8	2.75±0.082
	12.5	40± 9.6[b]	50.0±1.16	2.00±0.315[c]
	25.0	21± 3.0[d]	57.0±1.05[b]	1.20±0.089[d]
Chenodeoxycholic acid	50	86± 1.2[b]	36.1±3.5[b]	3.11±0.245
	100	93± 6.8[b]	22.0±4.4[d]	2.05±0.326[c]
	200	114± 5.4[d]	14.9±0.9[d]	1.71±0.130[d]
Deoxycholic acid	100	80± 9.8	37.9±1.06[d]	3.03±0.209
	200	74± 8.4	24.2±2.86[d]	1.79±0.366[c]
	300	63± 7.7	20.0±1.19[d]	1.26±0.300[d]
Cholic acid	250	135± 3.7[d]	22.9±1.43[d]	3.09±0.103
	500	144± 9.9[d]	15.2±1.68[d]	2.18±0.150[d]
	750	187±12[d]	8.6±0.9[d]	1.61±0.155[d]
Dehydrocholic acid	300	185±16[d]	14.3±0.9[d]	2.64±0.151[c]
	600	181±14[d]	10.5±0.6[d]	1.90±0.125[d]
	1200	178±4.8[d]	6.1±0.8[d]	1.09±0.117[d]

[a] Bile acids were injected intravenously 20 min after the administration of amaranth (AM; 300 μmol/kg i.v.) then bile was collected for 20 min. The values are the means ±SE obtained in 4—6 rats

[b] $p < 0.05$; [c] $p < 0.01$; [d] $p < 0.001$

Table II

Effect of bile acids on the respiration of isolated liver mitochondria[a]

Bile acid	Concentration $\times 10^{-5}$ M	Relative activity (%) of oxygen consumption[b] stimulated by		
		glu-mal	DNP	ADP
Lithocholic acid	1.66	225	79	75
	3.33	930	47	54
	5.00	720	14	36
	6.66	360	6	18
	10.0	95	1	9
Chenodeoxycholic acid	3.33	183	96	94
	8.33	467	77	86
	16.6	1210	41	57
	33.3	820	6	49
	50.0	280	1	27
	66.6	173	0	9
Deoxycholic acid	3.33	100	90	95
	8.33	247	63	86
	16.6	1060	38	68
	33.3	1150	16	55
	50.0	416	4	32
	66.6	250	1	19
Cholic acid	33.3	240	77	91
	66.6	1290	65	68
	100	1000	48	59
	133	863	33	36
	200	555	5	23
	266	353	1	16
Dehydrocholic acid	66.6	120	75	90
	133	95	66	67
	200	100	49	56
	266	113	39	47
	400	73	22	39

[a] The values are the means of three determinations

[b] Relative activity $= \frac{O_2 \text{ consumption after the addition of bile acids}}{O_2 \text{ consumption before the addition of bile acids}} \cdot 100$

Discussion

Bile acids are known to inhibit various active transport processes. For example, bile acids have been shown to inhibit hexose [4, 15, 30] amino acid [4] and ionic transport [28] in the intestine, ionic transport in gastric mucosa [23] and hippurate and iodipamide uptake in the kidney, uvea and chorid plexus [1]. These inhibitory effects are nonspecific [1, 4]. Trihydroxy bile acids are less potent inhibitors than the mono- and dihydroxy bile salts [1, 15]. The latter are known to produce cholestasis [7, 8, 21, 25] and cirrhosis [18, 19].

The excretion properties of AM permitted a selective investigation of the inhibitory effects of bile acids on the hepatobiliary transport. The present study has shown that bile acids are also able to inhibit this active transport process (Table I). The inhibitory potencies of bile salts, however, differ considerably from each other. If the inhibitory potency of the synthetic triketo bile acid DHCA is taken as unity, then the corresponding value for the monohydroxy bile acid LCA will be 44, for the dihydroxy bile acids CDCA and DOCA 4.4 and 3, respectively, whereas for the trihydroxy bile acid CA approximately 1. This indicates that hydroxylation of the steroid nucleus in the bile acid molecule reduces its inhibitory effect on hepatobiliary transport.

The bile acids investigated also affected the respiration of isolated liver mitochondria (Table II). The results presented here are compatible with the observation that bile salts inhibit electron transport and, with the exception of DHCA, uncouple oxidative phosphorylation in mitochondria [26]. It is interesting to note that the sequence of bile acids as stimulants of the state 4 respiration or as inhibitors of DNP- or ADP-stimulated mitochondrial respiration, directly correlates to their inhibition of biliary AM excretion. It is difficult to determine if the effect bile acids have on hepatic mitochondrial function is responsible for the decrease in biliary transport of organic anions. Sulfobromophthalein, a potent inhibitor of mitochondrial respiration [3, 22, 24] strongly inhibits in an uncompetitive fashion the biliary excretion of its glutathione conjugate [11]. The maximal biliary excretion rates of several nonmetabolized cholephilic organic anions are inversely correlated to their toxic potencies for mitochondria [12]. In addition, the cholephilic organic acids that are toxic to mitochondria are more potent inhibitors of the biliary excretion of AM than those exerting only a negligible effect on mitochondrial functions (unpublished observation by the authors). The uncoupler dinitrophenol depresses the biliary excretion of bilirubin by the isolated perfused liver [2]. The inhibitory effect of bile acids on transport of sugars by the intestine has also been suggested to be due to their effect on oxidative phosphorylation [5]. Bile acids have been shown to decrease oxygen consumption of intestinal mucosal homogenizates [5] and jejunal strips [28] as well as tissue ATP

levels [5]. All these observations support the concept that the deleterious effect of bile acids on liver mitochondria may play a part in their inhibition of the hepatic transport of AM. It is interesting to note that despite some conflicting results [5, 16] bile acids are believed to decrease the activity of Na^+-K^+-activated ATPase in different preparations. Moreover, the concentrations of bile acids (I_{50}) that found to inhibit Na^+-K^+-Mg^{++}-activated ATPase of hepatic microsomal membranes [27] are similar to those shown here for ADP-stimulated mitochondrial respiration. Therefore, it appears that the inhibitory effect of bile salts on mitochondrial respiration can be regarded as a part of their general effect on membranes.

Since bile acids and AM are higly cholephilic substances, a competitive inhibition of AM transport into the bile by bile salts could also be anticipated. As shown in Fig. 1, this probably occurred only with CA and DHCA, whereas mono- and dihydroxy bile salts exhibited uncompetitive inhibition.

In conclusion, it appears that the inhibition of the hepatobiliary transport of AM by LCA, CDCA and DOCA is due, at least in part, to the inhibitory effect of these bile acids on mitochondrial functions. In contrast, the effect that CA and DHCA have on mitochondria does not seem to manifest itself *in vivo*. The inhibitory effect these bile salts have on the biliary excretion of AM may result from a competition for the transport system rather than a toxic depression of the hepatobiliary transport. In addition, the high potency of LCA to interfere with mitochondrial functions may also play a role in its cholestatic effect.

Acknowledgement

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Recensiones

H. AUTRUM (Ed.)

Vision in Invertebrates. B.

Invertebrate Visual Centers and Behaviour I.

Handbook of Sensory Physiology. Vol. VII/6B.

Springer Verlag, Berlin—Heidelberg—New York 1981. 629 pages with 319 Figures and 10 Tables, price DM 270,—; approx. US $ 159.30

This volume is the second one of a series of three. Its four chapters are,

1. Neuroarchitectures serving compound eyes of Crustacea and Insects (N. J. STRAUSSFELD and D. R. NÄSSEL).
2. Neural principles in peripheral visual systems of Invertebrates (S. LAUGHLIN).
3. Polarization sensitivity (T. H. WATERMAN).
4. Optics and vision in Invertebrates (M. F. LAND).

The first chapter summarizes the basic principles of nerve cell organization in crustacean and insect optic lobes. It describes the basic arrangements of single cells in the three main synaptic regions (the lamina medulla and lobula complex, illustrates species differences of cell relationship and shows that a limited number of neuron types is involved in specific geometries. The use of cobalt impregnation makes the structure of the optic lobes look more simple than has been described previously. It is shown how single nerve cell populations contribute to simple arrangements of parallel retinotopic pathways and how these are sorted out to specific regions of the lateral midbrain and thence to descending pathways. The structural–functional correlates of the neurons, too, are summarized.

The second chapter deals with neural principles, concerning the organization of cellular components that assimilate and process optical information from the environment, to help the animal to maintain a favourable position within its surrounding. The generation of visually mediated behaviour is analyzed, summarizing the data concerning the adaptation of structure to function in the visual system of invertebrates. It is concluded that the neural principles of invertebrate visual systems are indeed the principles of vision in general.

The third chapter describes the sensitivity of invertebrates to the polarization of light. This phenomenon is still imperfectly known, but the author gives a definition and exact description as well as an example of this kind of sensitivity. The polarization sensitivity of crustaceans, insects, arachnids and cephalopod molluscs is discussed and a comparison is made with the same sense of vertebrates.

The fourth chapter compares various types of optical systems and describes their evolution. Here the various physical factors that determine and limit the performance of eyes are also discussed.

In the book author and subject indexes are given. The quality of the printing including microphotos, drawings, etc. is excellent. Specialists in physiology, biophysics, biochemistry, behavioural physiology, genetics, or zoology will undoubtedly find the book important and useful. It is highly recommended to university departments of physics and biology.

K. S.-RÓZSA

H. BOTHE and A. TREBET (Eds)

Biology of Inorganic Nitrogen and Sulfur

Springer Verlag, Berlin—Heidelberg—New York 1981. 384 pages with 144 Figures, price DM 89,—; approx. US $ 46.80

Nitrogen and sulphur compounds are continously synthetized, degraded and converted into other forms in nature. There are many similarities in the principal problems and basic mechanisms of the biology of these materials. Many details are not yet understood and hence form the subject of active investigations all over the world.

The book contains the most interesting results of both the nitrogen and sulphur cycle genetics and ecology of dinitrogen fixation, of assimilatory and dissimilatory nitrate and sulphate reduction and of ammonia and sulphide oxidation. We can find all the contributions to the conference held in May 1980 in Bochum, GFR, that discussed a research programme on the metabolism of inorganic nitrogen and sulphur compounds, supported by the Deutsche Forschungsgemeinschaft.

Among others, the following important chapters are to be found in the book.

Microorganisms involved in the nitrogen and sulphur cycles, nitrogen metabolism in plants, the assimilatory reduction of nitrate, dissimilatory nitrate reduction of dinitrogen fixation including some aspects of the physiology of dinitrogen fixation and its biochemistry, pathway and regulatory aspects of N_2 and NH_4^+ assimilation in N_2-fixing bacteria and other organisms. Assimilatory sulphate reduction, ecology and physiology of some anaerobic bacteria from the microbial sulphur cycle; sulphate uptake activation and dissimilatory sulphate reduction. Enzymatic mechanisms and the regulation of these metabolisms; *in vitro* association between nonlegumens and *Rhizobium;* the role of thioredoxin for enzyme regulation in *Cyanobacteria*, etc.

Figures and graphs complete the text and make it illustrative. For instance in one of the figures a polyacrylamide gel electrophoresis of gene products in shown encoded by wild-type and mutagenized fragments, in another we can see isolated membrane fragments of *Nitrobacter* cells with high, low and no nitrite oxidase activity, in others there are some new metabolism pathways, etc.

The book presents the latest results and knowledge about the above elements of plant life; and for this reason it is a significant handbook in plant physiology.

G. VERZÁR-PETRI

T. SZABÓ and G. CZÉH (Eds)

Advances in Physiological Sciences. Vol. 31.

Sensory Physiology of Aquatic Lower Vertebrates

Pergamon Press—Akadémiai Kiadó, Budapest 1981, pp. 1—285.

This volume is one of the series containing the proceedings of the 28th International Physiological Congress; the papers were discussed at a Satellite Symposium organized in Keszthely (Hungary) 21—22 July, 1980. The programme of the symposium was focussed on the electrosensory and electromotor systems in fish. The papers cover a wide range of knowledge from anatomy to ethology in this comparatively new branch of neurobiology.

The reader will find papers dealing with morphological, developmental and physiological aspects of electroreceptors as well as with certain histochemical and functional problems of the CNS of *Teleosts*. The transmitter profile between sensory cells and afferent nerve fibres and in the optic tectum of *Teleost* species is also discussed.

Several papers demonstrate new details of the medullary pacemaker nuclei which control the electric organ discharge, proving with HRP labelling the existence of pacemaker and relay cells. A large variation in the organization of the electromotor system in different species is described. Two papers deal with social behaviour of strongly electric fish.

In a whole the volume gives useful information in the sensory physiology of aquatic lower vertebrates. It can be recommended for the laboratories dealing with the above topic, but will also be useful for universities teaching sensory physiology.

K. S.-RÓZSA

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РЕЗЮМЕ

ВЛИЯНИЕ УВЕЛИЧЕНИЯ ВЫДЕЛЯЮЩЕЙ ПОЧЕЧНОЙ МАССЫ НА ДЕЯТЕЛЬНОСТЬ ПОЧЕК В УСЛОВИЯХ БЛОКИРОВАНИЯ АЛЬФА РЕЦЕПТОРОВ

Х. ТОСТ, Р. ХЕРЦЕГ и ДЬ. КЁВЕР

В экспериментах на наркотизированных собаках мы изучали влияние увеличения почечной массы, принимающей участие в процессах выделения, на деятельность почек. 10 экспериментальных животных не получали никаких фармакологических препаратов, другим 10 животным вводили препараты, блокирующие альфа-рецепторы.

В группе без лекарственных средств увеличение выделяющей почечной массы (трансплантация пары изолированных почек, происходящих от другого животного, на шею экспериментального животного), в почках *in situ* сопровождалось выраженным увеличением клиренса ПАГ и небольшим уменьшением клиренса инулина, и в то же время незначительным снижением выделения мочи и значительным уменьшением выделения натрия. Диурез и натриурез четырех почек соответствовали количествам мочи и натрия, выделяемых прежде двумя почками *in situ*.

В группе животных с блокированными альфа-рецепторами после увеличения почечной массы клиренсы ПАГ и инулина в почках *in situ* не изменились, уменьшение выделения мочи и натрия было таким же, как в предыдущей экспериментальной группе. Диурез и натриурез четырех почек и в данном случае соответствовали количествам мочи и натрия, выделяемых почками *in situ* до увеличения выделяющей почечной массы.

На основании результатов наших экспериментов создается впечатление, что, наблюдаемое при увеличении выделяющей почечной массы, уменьшение выделения в почках *in situ* натрия не есть следствие гемодинамических изменений, наступающих вследствие нагрузки на кровообращение, а является результатом какой-то быстрой, предположительно гуморальной, регуляции.

Мы считаем, что за уменьшение выделения натрия, наблюдаемого в почках *in situ* при возрастании выделяющей почечной массы, ответственным является повышенное — благодаря четырем почкам — потребление т. н. натриуретического фактора(-ов), что приводит к снижению уровня натриуретического гормона.

ВЛИЯНИЕ ГИСТАМИНА И БРАДИКИНИНА НА СОСТАВ ТКАНЕВОЙ ЖИДКОСТИ И ЛИМФЫ В ЗАДНИХ КОНЕЧНОСТЯХ КРОЛИКА; АРГУМЕНТ В ПОЛЬЗУ СУЩЕСТВОВАНИЯ ДВУХ ПРОСТРАНСТВ В ТКАНЕВОЙ ЖИДКОСТИ

ДЬ. САБО и Ж. МАДЬЯР

Собирали лимфу и тканевую жидкость задней лапы кролика, до и после внутривенной инфузии гистамина (2,5 мкг/кг/мин и 10 мкг/кг/мин) и брадикинина (0,4 мкг/кг/мин). Перед инфузией концентрация белка была выше в тканевой жидкости, чем в лимфе. У контрольных животных, за двухчасовой экспериментальный период, концентрация белка в лимфе увеличилась, а в тканевой жидкости уменьшилась. В контрольных опытах лимфоток снизился, не изменился он достоверно также после введения гистамина, брадикинин же у большинства животных (у 14 из 20) вызвал значительное увеличение лимфотока. Как

гистамин, так и брадикинин существенно увеличили количество белка и молчно-кислой дегидрогенезы (LDH), переносимых лимфой. У тех животных, у которых вазоактивные вещества не оказывали влияния на лимфообращение, значительно возросла активност LDH в лимфе, но в тканевой жидкости активность лактат-дегидрогеназы существенно не изменилась. В тех случаях, когда параллельно снижению лимфотока, уменьшалась активность лимфатической LDH, в тканевой жидкости это не сопровождалось соответствующими изменениями. На основании результатов настоящих экспериментов можно сделать вывод, что интерстициальное пространство состоит из двух отделов *(compartment)*. Лимфатические сосуды непосредственно дренируют только периваскулярный отдел, и лифа по сути является вновь образованным микроваскулярным фильтратом. Второй отдел межклеточной жидкости окружает клетки и соединительнотканные волокна и параллельно соединен с первым отделом.

ВЛИЯНИЕ АДРЕНАЛИНА И РОДСТВЕННЫХ СОЕДИНЕНИЙ (ДОФАМИН, ИЗОПРОТЕРЕНОЛ, ЭФЕДРИН) НА ПОТРЕБЛЕНИЕ САХАРА ОДНОКЛЕТОЧНЫМ ОРГАНИЗМОМ *TETRAHYMENA* И СОДЕРЖАНИЕ ГЛИКОГЕНА

Ж. ДАРВАШ, ДЬ. ЧАБА и В. ЛАСЛО

Особи штамма GL *Tetrahymena pyriformis*, под влиянием 10^{-3} М адреналина, дофамина или изопротеренола повышают потребление сахара, тогда как под воздействием эфедрина потребление ими сахара не изменяется. Уровень гликогена под влиянием 10^{-6} М адреналина повышается и без добавления экзогенного сахара, но у родственных эфедрину соединений этот эффект отсутствует. В то же время в присутствии экзогенного сахара все три родственных соединения в одинаковой степени повышают уровень гликогена, хотя это действие у адреналина является более слабым.

ПРЕДПОЛАГАЕМАЯ РОЛЬ L-АРГИНИНА В МОБИЛИЗАЦИИ ЭНДОГЕННОГО ФОРМАЛЬДЕГИДА

А. ЧИБА, Л. ТРЕЙЗЛ, Э. ВАРИ, ДЬ. ТЕЙГЛАШ и Х. ГРАБЕР

Авторы считают, что желаемое количество эндогенного формальдегида внутри организма обеспечивается деятельностью биологического регуляционного механизма. По пихмнению, регулирование связано с L-аргинином, непосредственно с энзимом аргиназой. Аргинин спонтанно реагирует в реакции равновесия с формальдегидом, во время образования производного гидроксиметила, и таким образом способен на мобилизирование формальдегида в желаемом направлении. Авторы предполагают, что метилоловое производное аргинина обладает способностью тормозить клеточное деление.

РАЗНЫЙ ЭФФЕКТ ИЗМЕНЕНИЙ СОКРАТИТЕЛЬНОЙ АКТИВНОСТИ И АКТИВНОГО ИОННОГО ТРАНСПОРТА НА NADH-ФЛУОРЕСЦЕНТНОСТЬ И ПРОДУКЦИЮ ЛАКТАТА В ГЛАДКОЙ МУСКУЛАТУРЕ МАТКИ

Г. РУБАНИ, А. ТОТ и А. КОВАЧ

Целью экспериментов было выяснить, какое влияние оказывают изменения сократительной активности и активного ионного транспорта на флуоресцентность NADH, продукцию молочной кислоты и изометрическое напряжение изолированной матки крысы и кролика. Повышение (127 мМ K^+) или торможение (раствор без Ca^{2+}) сократительной активности снижало (окисление NADH) или соответственно, увеличивало (редукция NADH) интенсивность тканевой флуоресцентности. Стимулирование натриево-калиевого насоса путем удаления Ca^{2+} вызывает в свежей мышце или нагруженной натрием (предварительным введением 30 мМ KCl) мышце окисление NADH, которое можно было ингибировать оуабаином (10^{-3} М). Активирование или ингибирование натриево—калиевого насоса все-

гда вызывали параллельно либо повышение продукции молочной кислоты, либо ее понижение, независимо от актуального сократительного состояния. Результаты экспериментов показывают, что 1. поверхностная NADH-флуоресцентность является методом, пригодным для прослеживания изменений обмена веществ в матке, и 2. аэробный гликолиз и натриево-калиевой транспорт тесно связанные друг с другом процессы в миометрии.

СЕЗОННЫЙ ЦИКЛ ФУНКЦИОНИРОВАНИЯ ГОНАД, ЩИТОВИДНОЙ ЖЕЛЕЗЫ И НАДПОЧЕЧНИКОВ У ГРАЧЕЙ *(CORVUS FRUGILEGUS)*

П. ПЕЦЕИ и ДЬ. ПЕТЕШ

Мы изучали сезонную деятельность половых желез, щитовидной железы и надпочечников у самцов и самок грачей, собирая птиц в природных местах обитания, в характерные периоды их жизненного цикла. В период формирования пар возрастание концентрации тестостерона в плазме у самцов вначале отражало увеличение веса семенников. Одновременно с тестостероном повышался также уровень тироксина. Содержание празменного кортикостерона увеличивается с «фазовым запозданием» — спустя один месяц. В конце воспроизводительного цикла сначала наступает сильное снижение уровня тестостерона, а затем, с полумесячным запозданием, уменьшение веса семенников. Перед послебрачной линьки и во время нее в плазме наблюдается высокий уровень трийодтиронина и тироксина, а также кортикостерона. В осенне-зимний период покоя концентрация половых стероидных гормонов, гормонов щитовидной железы и уровень глюкокортикостероидов в плазме показывают низкие значения. У самок в период устройствапочинки гнезда фолликулы находятся в фазе роста (белые и маленькие желтые фолликулы), в плазме в первую очередь возрастает уровень прогестерона и, в меньшей степени, эстрона и тестостерона. Одновременно сильно повышается также уровень тироксина и кортикостерона. В период спаривания яичники характеризует наличие крупных желтых фолликулов. Концентрация прогестерона и тестостерона в плазме уменьшается, уровень эстрогена, однако, не изменяется. Значительно уменьшается содержание тироксина и кортикостерона, в то же время концентрация трийодтиронина в небольшой степени повышается. В период кладки яиц (периовуляционная фаза) повышение уровня прогестерона и эстрона сопровождается повышением уровня тироксина и кортикостерона. В период высиживания птенцов концентрация всех половых стероидов, гормонов щитовидной железы и концентрация кортикостерона в плазме снижаются. Наблюдается быстрая инволюция яичника. До и во время послебрачной линьки количество половых стероидов продолжает уменьшаться, в то же время сильно возрастает содержание тироксина и кортикостерона. Основным моментом, обусловливающим формирование уровней гормонов, характерных для отдельных фаз сезонного цикла, у самцов кажется тирео-адренокортикальный синергизм, а у самок синергизм следующих пар гормонов: прогестерон — тироксин, прогестерон — кортикостерон и тироксин — кортикостерон.

ЭЛЕКТРОФИЗИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ, ВЫПОЛНЕННОЕ НА МАКСИЛЛЯРНЫХ ЩУПИКАХ СТРАНСТВУЮЩЕЙ САРАНЧИ.

I. ОБЩИЕ ОСОБЕННОСТИ РЕЦЕПТОРНЫХ ОТВЕТОВ НА РАЗДРАЖЕНИЕ ХЛОРИСТЫМ НАТРИЕМ

И. ВАРАНКА

Было показано, что, на раздражение хлористым натрием в растворах различной концентрации, отвечают 1, 2 или 3 рецепторные клетки сензилл, среди которых, однако, одна клетка всегда «доминирующая». Частота 1 или 2 второстепенных клеток ниже, адаптация более быстрая. Реакции доминирующих клеток на повторение раздражения даже тем же веществом, в той же самой концентрации очень изменчивы. При повышении концентрации с 0,01 моля в литре до 1,0 моля в литре начальная частота может увеличиваться или уменьшаться, но и при промежуточной концентрации частота тоже может быть максимальной или минимальной. Все это указывает на слабую специализацию рецепторных клеток. Индивидуальные особенности рецепторных клеток затушевываются при усреднении ответов достаточного числа клеток, индивидуальные отклонения уравниваются.

ДЕЙСТВИЕ ЖЕЛЧНЫХ КИСЛОТ НА БИЛИАРНУЮ ЭКСКРЕЦИЮ АМАРАНТА И НА ПРОЦЕСС ДЫХАНИЯ В МИТОХОНДРИЯХ ПЕЧЕНИ

З. ГРЕГУШ, Ф. ВАРГА и Э. ФИШЕР

Нами исследовалось влияние желчных кислот — литохолевой, хенодеоксихолевой, деоксихолевой, холевой и дегидрохолевой — на билиарную экскрецию амаранта и дыхание изолированных митохондрий печени в экспериментах на крысах. Желчные кислоты снижали билиарную экскрецию амаранта, усиливали в условиях *in vitro* дыхание митохондрий, измеренное в присутствии глютамат-малата, и подавляли потребление кислорода, стимулированное DNP и ADP. ID_{50} значения (мкмоль/кг, внутривенно) торможения, относящиеся к секреции амаранта, и ID_{50} концентрации торможения (х 10^{-5} М), относящиеся к стимулированному ADP митохондриальному дыханию, были следующими для разных желчных кислот: литохолевая кислота: 16 и 3,3, хенодеоксихолевая кислота: 160 и 24, деоксихолевая кислота: 230 и 31, холевая кислота: 680 и 105, дегидрохолевая кислота: 700 и 260. Литохолевая, хенодоксихолевая и деоксихолевая кислоты затормаживали не компетитивным образом билиарную экскрецию амаранта.

Результаты настоящих экспериментов указывают на то, что токсическое действие на митохондрии литохолевой, хенодеоксихолевой и деоксихолевой кислот может играть роль в том, что эти желчные кислоты оказывают тормозящее влияние на билиарную секрецию амаранта.

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Physiologia—Pathophysiologia

ISOLATION, PROPERTIES AND P CONTENT OF THE HUMAN BRAIN MYOSIN

by

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KCl-, and NaCl-myosins were prepared from different parts of the central nervous system (CNS). Throughout these experiments P and lipid contents were higher in NaCl-myosins than in KCl-preparations. Both KCl-, and NaCl-myosins have increased lipid and P contents compared with skeletal muscle myosins.

When the specimens were separated by a molecular sieve, it was found by chromatographic technique on Sepharose 4B column that the cerebral and cerebellar myosins were composed of two fractions of different molecular mass while the brain stem and spinal cord myosins revealed only a single peak.

The myosin fractions' Ca-ATPase activity could be augmented by rabbit muscle actin. The myosin preparations developed filamentous systems and aggregates which could be shown by scanning electron microscopy.

All the CNS-myosin preparations could be phosphorylated; however, they were saturated to a different degree and were influenced by the presence or absence of serotonin.

The kinetic studies revealed that the phosphate saturation of the brain stem, cerebellar and cerebral myosins depended on the ATP concentration and incubation time.

The alkaline hydrolysates of lipid-free human brain myosin preparations contained amino acid phosphates, P-Arg, P-Lys and P-His in different amounts depending on their sources. In response to a phosphorylating mixture only the amount of P-Arg was elevated in the cerebral myosins, P-Arg and P-His in the brain stem preparations, and P-Arg, P-His and the amounts of unidentified compounds in the cerebellar ones.

An actomyosin-like complex from mammalian brain was first isolated by Puszkin et al. [20]. The complex showed superprecipitation with magnesium ions and ATP. The protein complex constituted approximately 1 to 2% of the total rat or cat brain protein. Subsequently, Puszkin et al. [21], Berl et al. [2] have shown that actomyosin is present in the synaptosomal fractions and as shown by Puszkin and Kochiwa [22], brain actin is enriched in the synaptosomal membrane fraction, while myosin molecules have been found mainly in synaptic vesicles. Burridge and Bray [5] identified in the brain of 12 to 14-day-old chick embryos heavy chains of myosin with a molecular weight of about 200 000 dalton: and light components of 17 000, 20 000 and 23 000 dalton. The heavy chains show major differences compared with those derived from skeletal, cardiac, platelet, and adult brain myosins.

The myosin molecules from all sources including the brain are morphologically indistinguishable as shown by ELLIOTT et al. [9]. They all have two globular heads attached to a tail the length of which does not differ by more than 10 nm from species to species.

ROISEN et al. [23] have demonstrated by the double antibody immunofluorescent method in cultured neuronal and glial cell lines that the cells have a three-dimensional surface network and an array of parallel filaments aligned with the major cellular axis. The myosin-rich filaments are found in the region of the actin filament system network. They have suggested that these proteins are capable of interacting to generate the motive force for the intracellular movements.

ASH [1] described the properties of myosin isolated from cultured glial cells of the rat brain. Therefore, it is beyond any doubt that the glial cells contain myosin and actin, too. The heavy subunit of glial myosin has a molecular weight of approximately 200 000 dalton while the light chain components have a molecular weight of 17 000 and 19 000 dalton; 0.2 mg myosin is gained from 5 g of cultured cells.

The aim of these experiments is to obtain sufficient amounts of myosin from the human cerebral cortex and from other parts of the central nervous system (CNS) with an extraction solution containing KCl or NaCl and suitable for analytical comparison. Since it has been found that the myosin from rabbit skeletal muscle has higher a P content when NaCl is applied in the process of extraction and purification (FAZEKAS et al. [10]) we have made a distinction between KCl-myosin (with a lower P content) and NaCl-myosin (with higher P content).

Materials and Methods

The human brain was cleaned from the meninges and vessels at 0–4° and dissected into different parts. The brain was minced and weighed. (The majority of blood vessels and nerves remain inrolled on the spindle of the mincing-machine.)

Preparative work was done at 0–4 °C. Human brain myosin (HBM) was extracted from fresh minced brain, essentially by the method of BURRIDGE and BRAY [5]. The materials were homogenized either in buffer A (0.6 M NaCl, 8 mM $NaHCO_3$, pH about 8.7, 1.5 mM 2-mercaptoethanol) or in buffer B (0.6 M KCl, 8 mM $KHCO_3$, 1 mM dithiotreitol, pH about 8.6). We used the buffers A or B to compare the effect of Na^+ or K^+ on the P content of myosin (FAZEKAS et al. [10]). Buffers containing phosphates were avoided, similarly the precipitation by ammonium sulphate was omitted. The homogenate was stored for 30 min, then centrifuged at 16 000 g for 30 min; the extraction was repeated (3 hours) and the extract was centrifuged again.

The supernatants were combined and the crude myosin was flocullated by dialysis against distilled water changed twice, then followed by further dialysis against 40 mM NaCl or KCl whilst the pH was maintained at about 7.2 with 3 mM $NaHCO_3$ or $KHCO_3$. The precipitate was pooled by centrifugation at 16 000 g for 20 min (in the second part of the study 150 000 g for 150 min was used to enhance effectiveness), followed by washing, the material was resuspended in 20 vol of 40 mM NaCl or KCl and sedimented by centrifugation again as before. The myosin precipitate was dissolved in a small volume of 2 M NaCl or KCl, and 2 mM 2-mercaptoethanol. The pH was adjusted to 7.6 with $NaHCO_3$ or $KHCO_3$, while the

salt concentration to 0.6 M NaCl or KCl. The opalescent solution was then centrifuged at 16 000 g for 30 min.

The supernatant solution was precipitated again by dialysis and the precipitation was dissolved to a suitable volume as described above. Since brain myosin contains more lipid and nucleic acid than skeletal muscle myosin, these were removed by treatment with DEAE-cellulose (DE_{32}, 0.2 g suspended in 10 ml of 5 mM Tris-HCl buffer, pH 7, adding 0.1 ml suspension to 1 ml myosin), followed by centrifugation at 16 000 g for 30 min. The supernatant was then ultracentrifuged again at 150 000 g for 90 min to remove actin contamination (uc-myosin). This supernatant solution (3–5 ml containing maximum 70 mg protein) was trasferred to a column (1.8 × 70 cm) of Sepharose 4B equilibrated and eluted with buffer A or B. The flow rate was approximately 15–18 ml/h.

The protein content was determined by the biuret micro method (Goa and Scan [14]), and by absorption measured at 225 and 260 nm, and the dry weight of salt- and lipid-free protein was measured gravimetrically at the end of the procedure and after protein dehydration at 105 °C for 5–6 h.

The RNA contamination was traced in gel-filtrated samples on the basis of their ribose content determined by the orcinol method of Schneider [24], while the lipid-P, P content in lipid-free samples, and the total P content were determined in the inorganic residue gained by combustion with cc HNO_3, using the method of Fiske and Subbarow [12], except that the final reduction was reached by the addition of ascorbic acid according to Lowry et al. [17].

The HBM may be phosphorylated with a phosphorylating mixture containing 0.1–0.3 mg myosin in 1.5 ml final volume, 25 mM Tris-HCl, pH 7.28, 6 M $MgCl_2$, 0.1 mM ATP, 10 μM $CaCl_2$ and 60 mM NaCl or KCl depending upon the nature of the myosin preparate.

Phosphorylation was started with Mg-ATP mixture and terminated with 2–3 vol of ice-cooled acetone in proper time. The myosin was then allowed to flocullate and was pooled by centrifugation. Non-covalently bound P and ATP was removed by washing six times with a mixture containing 20 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 8 mM $NaHCO_3$, 1 mM EGTA, applied in 2 vol ethanol and finally without ethanol, followed by lipid extraction twice (Chl–MeOH, 2:1 by volume), and then the P content was determined in the samples and the control again.

The properties of covalently bound P were studied in the hydrolysates of lipid-free myosin. The fatty acids and derivates of cholin and ethanolamine phosphates interfere with the chromatographic separation of amino acid phosphates. The alkali-stable amino acid phosphates were obtained by a procedure of hydrolysis in 3 M KOH, at 105 °C for about 10 h in sealed "Pyrex" glass tubes. The details of the techniques and analytical methods necessary for the elimination of Si-background dissolved from the ampulles have been published in a previous communication (Fazekas et al. [11]). The alkali-stable amino acid phosphates were separated on a column (0.9 × 8 cm) of Dowex 1 X8 by a method of linear gradient chromatographic elution. The hydrolysate was loaded onto the column at a low concentration of KOH (0.01 M), and elution was performed using a stepwise linear $KHCO_3$ gradient up to 1 M. To determine the P content of the fractions, 0.5 ml of effluent from each fraction was added to a reaction mixture containing 0.5 ml ammonium molibdate reagent, 1.25 ml distilled water, 0.25 ml of 1% ascorbic acid, the H_2SO_4 concentration being 1 N. The inorganic P reacts immediately, while covalently bound Ps after release in their hydrolytic sequence of P-Arg, P-His, and their secondary hydrolytic products develop blue colour after hydrolysis, too. The intensity of the blue colour is read at 720 nm at a proper time similarly to their standard curves.

The specific reactions were applied for the trace of basic amino acids in the collected fractions. The colorimetric method of Gilboe [13] for arginine, that of Horinishi et al. [16] for histidine and that of Chinard [6] for lysine were applied; moreover their presence in freeze-dried samples was checked by paper- and TLC sheets as described in our previous report [11].

Results

During the isolation of our first preparations, we had to be careful not to make the same mistakes that had been made during the isolation of muscle myosins. Namely, we supposed the existence of a single individual myosin in CNS. Moreover, the yield of myosin that was obtained by the KCl-contain-

ing solution was less than the preparations of NaCl-myosin independently of whether it had been derived from the hemisphere or other parts of CNS. Nevertheless, the preparations still had a higher P content than the skeletal muscle myosin [10].

The yield of myosin and their P content from several brain preparations are demonstrated in Table I.

Table I

The yield of myosin preparations from the human brain

Subject		Material*	Mince (g)	Myosin preparations	Yield of purified myosin** (mg)	P content (mol/mol)	Ribose content (mol/mol)
age	sex						
KCl-myosin							
76	♀	lob. front. and occip.	150	non-gel-filtr. myosin	6.8	45	
86		right cerebr. hemisphere	505	uc-centr.	37	29	
				gel-filtr.	12	50	
65		hemisphere	830	uc-centr.	18.5	31	4.6
				gel-filtr.	10	51	trace
54		hemisphere	750	uc-centr.	16.9	29	12.5
				gel-filtr.	6.5	62	3
NaCl-myosin							
33	♀	hemisphere	849	uc-centr.***	44	67	15
				gel-filtr.	10.5	89	3
67		hemisphere	1050	uc-centr.	31	90	35
				gel-filtr.	15	158	5
82	♀	left cerebr. gray mat.	239	uc-centr.	12	95	12
				gel-filtr.	3.8	130	2

* The majority of myosin originated from the gray matter, since the process was tried from white matter and an insignificant myosin was only yielded.

** The data are related to dry weight of salt-, and lipid-free proteins.

*** Using KCl/NaCl in mol/mol ratio in the extracting solution.

It is obvious from these data that the HBMs have a higher P content than skeletal muscle myosin. The RNA contamination may be reduced to a minimum by DEAE-cellulose treatment and gel filtration.

There is also a higher lipid and P-lipid content in HBMs derived from different parts of the CNS compared with the muscle myosin; the highest P-lipid values were found in the NaCl-myosin, these are pooled and demonstrated in Table II A, B.

The myosin seems to be heterogeneous in the preparations made of the cerebral cortex and cerabellum, since they exhibited a double peak on exclusion chromatograms on Sepharose 4B gel (Figs 1 and 2).

However, a single myosin peak was gained from the material obtained from the brain stem and spinal cord (Figs 3 and 4). The double peak was

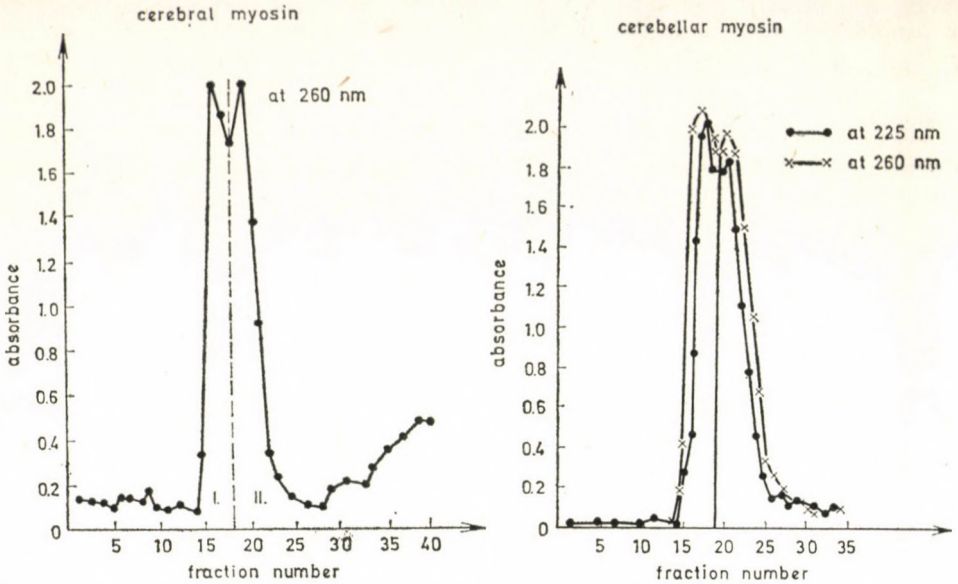

Fig. 1. Gel filtration of human cerebral myosin on Sepharose 4B column (2.3 × 63 cm). The column was equilibrated and eluated with 0.5 M NaCl, 8 mM $NaHCO_3$; 3.2 ml were collected into each tube. The tubes of the first fraction were collected as myosin separately by their peaks into two parts as I and II

Fig. 2. Gel filtration of cerebellar myosin on the same columns as in Fig. 1, except that 3.65 ml were collected into the tubes. Further details of the legend are the same as described in Fig. 1

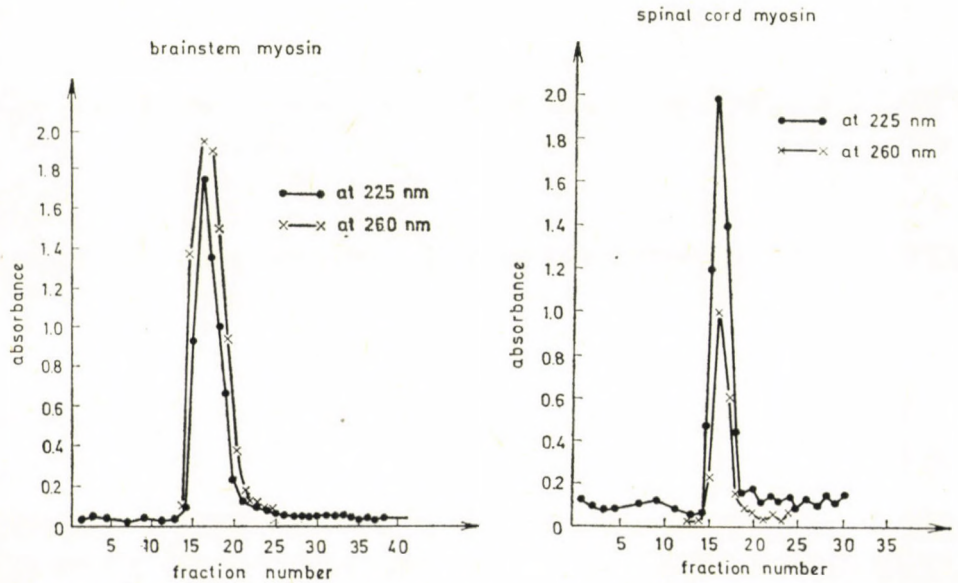

Fig. 3. Gel filtration of human brain stem NaCl-myosin on the same column as cerebral one. 4.7 ml fractions were collected. Tubes Nos 16—19 were collected as myosin

Fig. 4. Gel filtration of human spinal cord NaCl-myosin on the same column as the cerebral and brain stem myosins. 4.65 ml fractions were collected. Tubes Nos 16–19 were pooled as myosin

regarded as a mixture of two distinct myosins, therefore two separate fractions (I and II) were collected.

Table II shows that fraction I has a higher P content than fraction II in cerebral preparations. The question arises wheather the appearence of the double peak is a consequence of different molecular masses due to differences in covalently bound P, or lipid or P-lipid contents, or in all three components collectively.

Table II

Yield and properties of NaCl-*myosin from different parts of* CNS *of two subjects (A and B)*

A

	Mince (g)	Yield of purified protein (mg)	Total P content*	P content of lipid-free myosin*	Lipid-P*	Ribose content (mol/mol)
Hemisphere	665	37	289	265	42	2.4
Cerebellum	90	5.6	89	86	10	1.2
Brain stem	48	6.9	35	25	8	1.7

B

	Mince (g)	Gel-filtr. fractions**	Yield of gel-filtr. myosin* (mg)	P content of lipid-free myosin* (mol/mol)	Lipid P (mol/mol)
Left cerebral grey material	240	I	30	130	14
		II	19.6	83	19
Cerebellum	110	I	6	109	46
		II	5.5	154	53
Brain stem	69		3.8	116	20
Spinal cord	23		3.6	65.5	40

* Data are given mol P/mol of myosin and related to dry weight of salt- and lipid-free myosin preparations.

** I and II: see Figs 1 and 2; the tubes of gel-filtrated myosin are collected into "separate" fractions.

All gel-filtrated myosins are able to form filamentous systems as it is shown by a sample of cerebellar myosins (Figs 5 and 6).

The ultrafine section was made from a small part of the filamentous aggregate sectioned from a part of filaments embedded in Durcupane. The section shows the double heads and groups of heads, moreover the tail parts of myosin (Fig. 7). It seems that shrinking of the filaments may be avoided by this technique.

Ca-dependent ATPase activity in the samples of a high ionic strength medium (0.5 M KCl, pH 7.2) is between 4.18 and 6.0 nkat. mg^{-1}, i.e. 0.25–0.36 μmol Pi. mg^{-1}. min^{-1} similar to the results of BURRIDGE and BRAY [5].

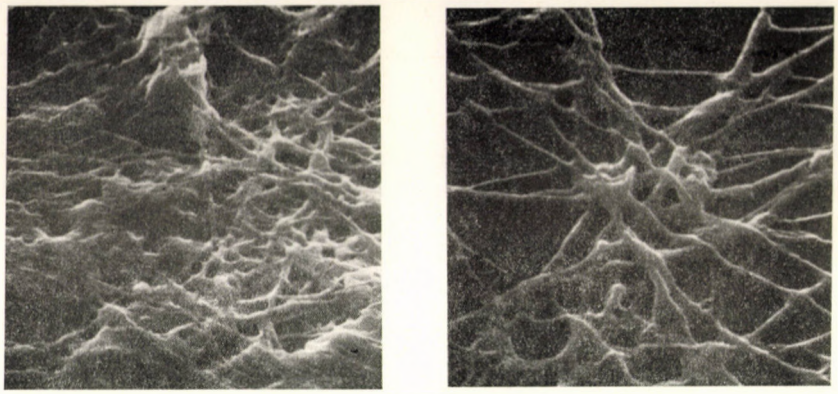

Fig. 5. SEM (scanning electron microscopic) figures of the filamentous aggregates of gel-filtrated cerebellar myosin. The formation of filamentous systems was induced by 0.2 mM $K[Au(CN)_4]$ solution stored at 0–4 C°, and postcontrasted with Au-treatment. Magnification $\times 3500$

Fig. 6. SEM figures of cerebellar myosin aggregates from fine structural filaments to illustrate the further details of the filamentous system. The preparation was postcontrasted with Au-treatment. Magnification $\times 9450$

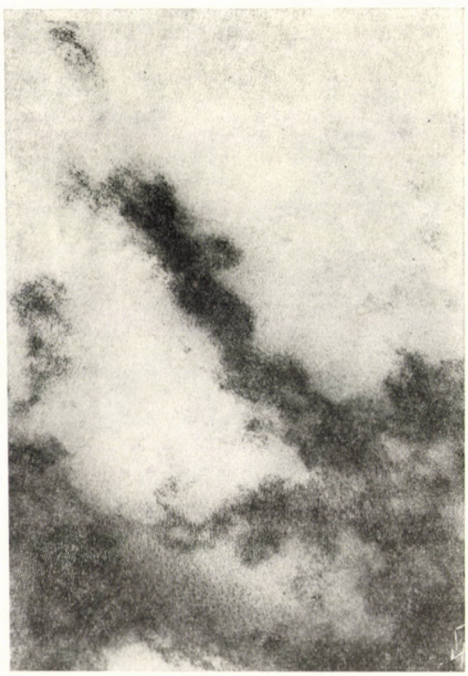

Fig. 7. TEM (transmission electron microscopic) structure of a segment of the filamentous system of the cerebral cortex myosin prepared from $K[Au(CN)_4]$-induced aggregates. The ultra fine section was made of Durcupane-embedded filaments, postcontrasted with Au-treatment. Note the double heads, groups of some heads and rod parts of myosin. Final magnification $\times 165\,000$

(nkat = nanokatal = nmol. s^{-1} by SI). The activity of HBMs may be enhanced about to two or ten times by rabbit muscle actin depending on the nature of myosin preparations.

In the further part of our study the chemical properties of P-containing compounds in myosin were investigated. In view of our previous observation that the phosphate contents of skeletal muscle myosin can be increased with ATP under suitable circumstances a similar phosphorylation process was performed with the respective brain myosins. Only KCl-myosins were prepared from three different parts of the brain (cerebral cortex, cerebellum, brain stem) of two subjects simultaneously and the phosphate incorporation was investigated in preparations to obtain more information about the existence of the three types of myosin. The samples were incubated in phosphorylating mixture, and also in the presence of serotonin (molar ratio serotonin: myosin = = 1000/1).

Due to its relatively high concentration in the brain stem and hypothalamus [19, 26, 27], and to its specific effect on synaptosomal preparations of the

Table III

Phosphorylation and effect of serotonin on the phosphorylation of human brain myosin
(data are related to mol P/mol myosin)

Type of sample	Samples of gel-filtrated preparations before phosphorylation		Samples incubated		
	total P**	lipid-P*	without Mg-ATP (control)	in phosphorylating mixture	in phosphorylating mixture + serotonin
KCl-myosin					
1. hemisphere	41.8	5	24	122	—
2. hemisphere	45.5ng	4.5	25	155	168
3. hemisphere a^+	30.7	4.5	16	83	102
b^+	50	8	33	94	168
NaCl-myosin					
4. hemisphere	516	47.6	220	575	571
5. hemisphere	391	30.7	268	645	495
6a) cerebral cortex I	315	14	130	330	566
II	83	19	55	47	72.5
b) cerebellum I	109	42.6	105	405	480
II	154	50	175	365	220
c) brain stem	116	19.8	47	9	237
d) spinal cord	71	40.5	10	108	255
7a) cerebral cortex	194	30.9	142	206	216
b) cerebellum	89	23.9	68	305	350
c) brain stem	117	52	56	22.5	30.7

ng, non-gel-filtrated (only ultracentrifuged sample)

a^+ and b^+ show two parallel preparations with minor modification in extraction solution of a^+ adjusted the pH to 6.6

* removed by Chl-MeOH mixture from acetone precipitated samples

** the samples contain the lipid content, too, showing the efficiency of the incubation and washing solution in the controls

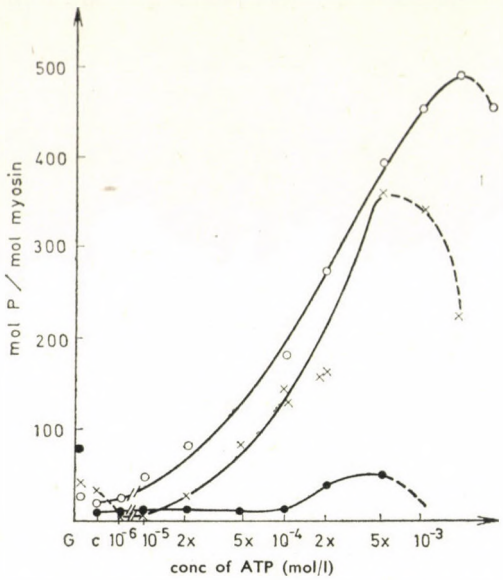

Fig. 8. Effect of ATP concentration on the phosphate saturation of gel-filtrated human brain myosins. The samples were incubated in the presence of increasing amounts of ATP in the same medium that was applied for incubation of the specimens in Table III. Incubation was performed at 30 °C and started with the addition of myosin samples and terminated with ice-cooled acetone at maximum phosphate saturation (see Fig. 9). The phosphate saturation of each specimen is represented in mol P/mol myosin versus ATP concentration. ·—·—· brain stem, x—x cerebellar, o—o cerebral myosin. G, gel filtrated, C control incubated without ATP

brain cortex [7] it was supposed that serotonin might have an effect on phosphorylation. The results are shown in Table III. This table shows that the P contents of NaCl-myosin preparations is higher than that of KCl-myosins.

All CNS preparations can be phosphorylated (except for the brain stem NaCl-myosin) in the presence of a phosphorylating solution, even if this contains ten times lower ATP than used for the phosphorylation of skeletal muscle myosin [10]. The data can be properly evaluated only if they are related to the controls since the incubation and the washing procedure further decrease the P contents of the gel-filtrated samples. The highest loss in P was found in the spinal cord myosin sample when it was incubated without ATP.

The data show that serotonin has an influence on the P preservation of most specimens.

The heterogeneity of the degree of phosphorylation in the KCl-, and NaCl-myosin preparations and the effect of serotonin have not been clarified, yet. The variety might depend on the origin of the KCl-, and NaCl-myosin preparations, furthermore on the subjects.

For a comparative analytical and kinetic study of phosphorylation higher amounts of HBMs preparations were required. At well defined stages

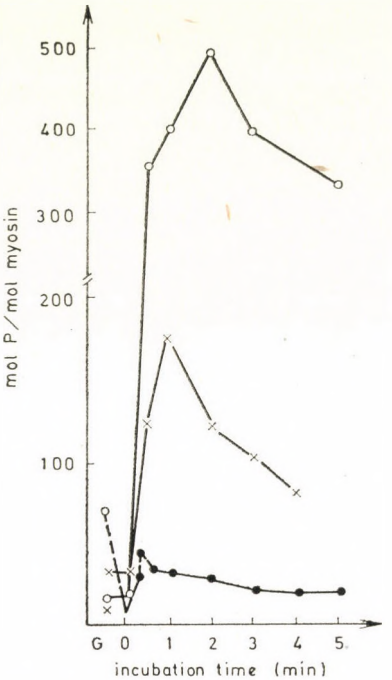

Fig. 9. Effect of incubation time on the phosphate incorporation of gel filtrated human brain myosins in the presence of 0.75 mM ATP in the incubation medium. The incubation was started with the addition of Mg–ATP mixture and terminated at increasing incubation intervals. The same symbols are used for the individual specimens as in Fig. 8. The phosphate saturation is designed and represented in mol P/mol myosin versus the incubation time

of the procedure a significant observation was made. The yield of myosin could be increased by about three times (shown compared with the amounts in Tables I—III) if the floculous precipitate of the first dialysate was sedimented by ultracentrifugation (150 000 g, 150 min; see Method). The efficiency of the modification is explained by the higher number and diversity of polar groups of HBMs related to the respective skeletal muscle myosins. As a consequence, they have higher solutibility resulting in finer precipitation which latter remains in solution mostly in rather dispersed formations.

Several pilot studies have shown that phosphate incorporation depends on the concentration of ATP in phsophorylating mixture and on the incubation time. The results of the dependence of phosphorylation upon ATP concentration are shown in Fig. 8. Incubation was terminated at different intervals (see Fig. 9). The obtained curves reveal very distinct deviations in phosphate saturation and ATP sensitivity depending on the origin of the preparations. The highest sensitivity for ATP concentration is shown by cerebral myosin. This preparation incorporates phosphate already at 10^{-6} M, while that of the

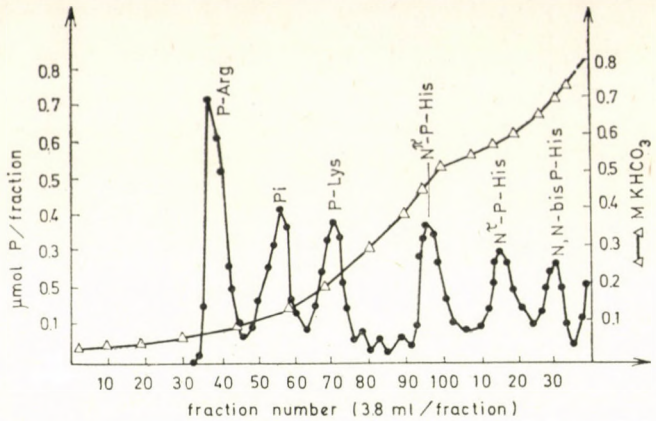

Fig. 10. Elution profile of synthetic alkali stable amino acid phosphates. The solution of 4 µmol samples was applied on Dowex 1 X8 ion exchange column (200–400 mesh, 0.9×6 cm) in 0.005 M KOH solution and separated with a linear step gradient method of chromatographic technique. The chromatogram was designed by the P content of the tubes having the phosphate determined in an aliquot of each tube. The elution profile was graphed on the basis of the chromatographic separation of each P-containing compound separately, then on that of the mixture of synthetic specimens

brain stem at a concentration of 10^{-4} M ATP. The highest incorporation was attained in cerebral myosin having the most phosphate binding sites. The findings in Figs 8 and 9 relate to preparations of patient No. 8. Similar results were obtained, with some differences, from parallelly investigated preparations (from patient No. 9).

When the incubation times were studied, differences were found again. The saturation of the brain stem preparation required the shortest incubation time. The phosphate saturation of this myosin terminated within 30 s, while the incorporation lasted in the cerebellar preparation about 1 min and in the cerebral cortex myosin about 2 min in the presence of 0.75 mM ATP (Fig. 9).

It was found that in the phosphorylated samples the highest amount of phosphate could be preserved when the incubation was terminated by ice-cooled acetone at maximum saturation. This provides a possibility for the study of incorporated phosphate in comparison with gel-filtrated samples (see Figs 8 and 9).

Further on an attempt was made to establish which type of amino acids the phosphoryl groups were connected with. For this special purpose the myosin samples were hydrolyzed, liberating the amino acid phosphates from peptid bonds by alkaline hydrolysis.

The alkaline hydrolysis simultaneously releases the free amino acids and alkali stable amino acid phosphates. Naturally, this procedure makes a possibility of secondary reactions as a small amount of secondary products is formed together with other hydrolytic derivatives. The elution profile of

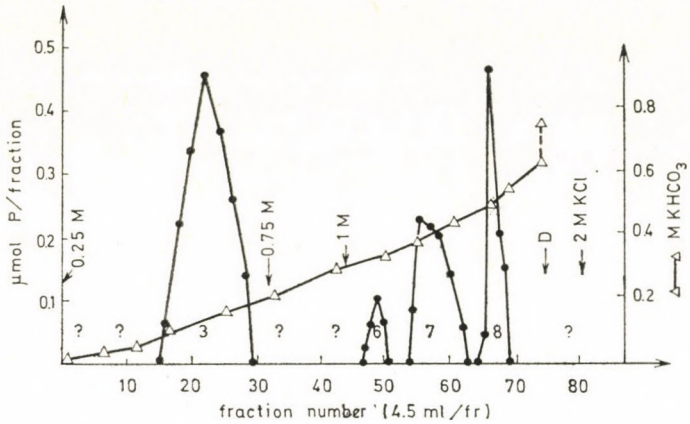

Fig. 11. Separation of the alkali stable amino acid phosphate from the hydrolysate of phosphorylated brain stem myosin. The hydrolysate was obtained from phosphorylated and lipid-free protein part (in 3 M KOH, 105 °C, 10 h) in a sealed "Pyrex glass" ampule, than diluted to 0.01 M KOH concentration and transferred onto Dowex I X8 column, 0.9×6 cm. The separation is performed with a linear step gradient chromatographic technique using a mixer chamber with a capacity of 160 cm^3 filled with 0.02 M $KHCO_3$ and the reservoir with 120 ml of 0.2 M $KHCO_3$ solution operated with an electromagnetic mixer in the course of elution. Then the $KHCO_3$ concentration was increased to 0.75 M and 1 M as indicated by the arrows. Finally, 1 M $KHCO_3$ was applied directly without mixing marked as D and 2 M KCl for the regeneration of column. The determination of P content in effluent tubes was undertaken on the basis of the specific molybdate test for phosphate considering the presence of Si-background (see [11]). The peaks are represented by the P content of tubes in µmol per fraction applying the same numbering for peaks as in Table IV. The brain stem myosin shows the least phosphorylated compounds related to cerebellar and cerebral preparations

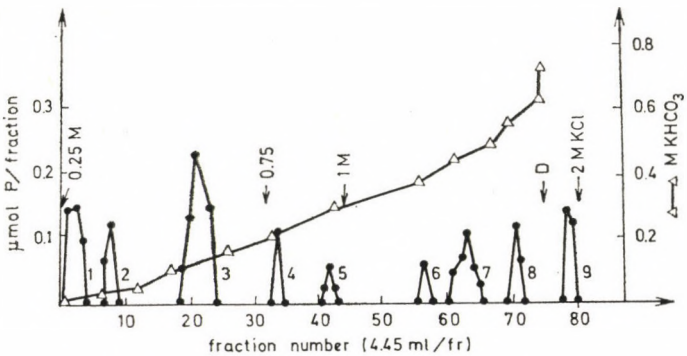

Fig. 12. Elution profile of amino acid phosphates of phosphorylated cerebellar KCl-myosin. The hydrolysate was obtained from the lipid-free protein part similar to brain stem myosin. For the rest of legends see Figs 10 and 11. The most phosphorylated compounds were separated from the cerebellar preparation confirmed by a concurrently investigated myosin of patient No. 9

synthetic amino acid phosphates and inorganic P are shown for the identification of phosphorylated amino acids of myosin on one of the chromatograms (Fig. 10).

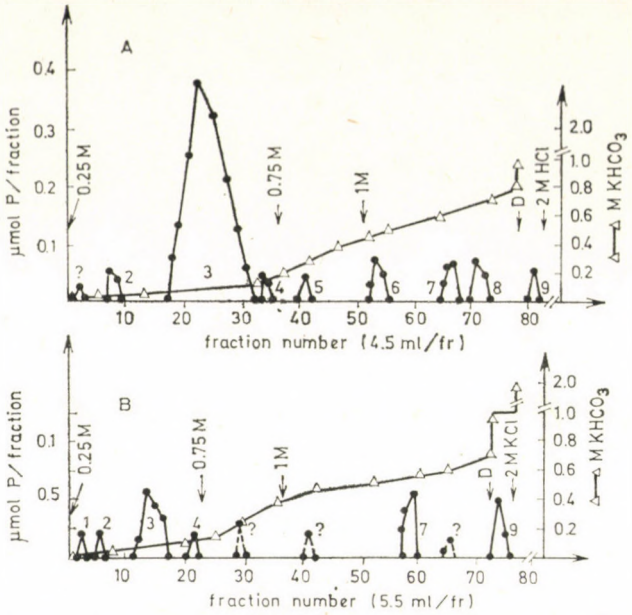

Fig. 13. Elution profiles of amino acid phosphates of cerebral phosphorylated (a) and unphosphorylated control (b) specimens. The control sample is represented by a different scale compared with the phosphorylated one (a). The most of phosphate was incorporated in P–Arg of cerebral gel-filtrated myosin (see Table IV). For the rest of the legends see Figs 10 and 11

The chromatogram of the individual amino acid phosphates was designed and constructed from those tubes which developed a blue colour with ammonium molybdate reagent (see Method).

The chromatographic profile of amino acid phosphates separated from the hydrolysate of brain stem myosin is shown in Fig. 11, furthermore the one obtained from one of the cerebellar samples is demonstrated in Fig. 12.

The hydrolysate of cerebral cortex myosin and its unphosphorylated sample are shown using different scales for the sake of comparison (Fig. 13).

Table IV summarizes the distribution of the phosphorylated compounds found in the hydrolysate of phosphorylated HBMs. Nine phosphorylated compounds were observed altogether so far in the myosins derived from muscle and non-muscle sources. This numbering is used in the table for peaks in order of elution, disregarding whether they have been identified or not, with the aim of summarizing the total P-containing compounds which have been observed in myosin up till now.

On the basis of experience gained at phosphorylation studies it might be assumed that probably more than 9 phosphorylated compounds exist as the hydrolytic and decarboxylated products Nos 1 and 2, respectively, are minor

Table IV

Distribution of amino acid phosphates in hydrolysates of phosphorylated human brain KCl-*myosin*

No. sample	Brain stem (phosphoryl).		Cerebellar (phsophorylated)		Cerebral cortex			
					(phosphoryl.)		(control)	
	μmol	%	μmol	%	μmol	%	μmol	%
1 ?	—	—	0.804	21.00	tr		0.045	6.95
2 ?	—	—	0.228	6.00	0.028	1.34	0.034	5.90
3 P-Arg	3.650	49.6	1.163	30.50	1.770	85.00	0.353	54.50
4 Pi	—	—	0.232	6.06	0.017	0.84	0.029	4.50
5 P-Lys	—	—	0.093	2.44	0.014	0.66	tr	
6 ?	0.757	16.20	0.113	2.96	0.075	3.60	tr	
7 N^{π}-P-His	1.632	22.9	0.588	14.60	0.103	4.95	0.153	23.40
8 N^{π}-P-His	1.106	16.1	0.214	5.60	0.115	5.35	0.019	2.92
9 ?	tr		0.414	10.80	0.032	1.49	0.050	7.70

components. In phosphorylated samples they are usually covered by fresh Nos 1 and 2 of higher amounts, which appear under the effect of phosphorylation in some myosins, from several sources.

Discussion

Experiments presented in this paper show that there might be three types of HBMs with distinct properties prepared from different parts of the CNS similarly to myosins of skeletal muscle varying according to their sources. Myosin heterogeneity has been revealed in examinations of BRAY [3] in cultured developing chicken neuronal myosin preparations. He has found that actin is about 8% and myosin only 0.5% of the total protein content of cultured neurocytes. Similarly, a rather low quantity of myosin has been found in adult tissues. Further myosin preparations have been isolated from other specific nervous sources: DRENKENHAHN et al. [8] from peripheral nerves, HESKETH et al. [15] from bovine retina, SCORDILIS et al. [25] from murine astrocytes, and MILLER and KUEHL [18] from rat glioma and cultured neuroblastoma derived from brain. Their shape, size and enzyme activity have been similar to muscle myosin, while the light chains of neuronal myosin were rather similar to that of smooth muscle myosins. However, there are some differences in the amino acid sequence of heavy chains that result in altered aggregative behaviour [4].

Our findings agree with those of WELLINGTON et al. [28], i.e. the preparation of brain myosins contain an abundance of lipids. We also found a higher lipid quantity in HBMs particularly in cerebral cortex myosin. Consequently, 480 000 dalton refers to the right molecular mass, and the combined molecular mass would be a fictitious value. A significant P-lipid part is closely adsorbed in gel-filtrated preparations, and for this reason it is supposed that an adequate lipid amount is an integrated part of the myosin molecule.

It has been experienced throughout these investigations that Na^+ has a specific influence on the preservation of P as it was found in the case of the skeletal muscle myosins [10]. Myosin was obtained with a higher P content from CNS in the presence of NaCl-containing solutions as compared to KCl-containing ones.

Myosins (including HBM, muscle and non-muscle myosins) may be induced by $K[Au(CN)_4]$ to form a filamentous aggregate system. Actin never produces the above-mentioned phenomenon.

It cannot be decided whether myosin in cerebral cortex and cerebellum is heterogeneous or there are two different types of it particularly in NaCl-preparations. Only the distinctly different P content seems to confirm this assumption. Systematic studies are required for the solution of this problem, since the myosin might have been derived from at least two different sources (brain cells and blood vessels).

However, the existence of three distinct myosins in human brain are confirmed by the phosphate saturation studies depending on the ATP concentration and incubation time (see Figs 8 and 9). The cerebral myosin is able to incorporate an enormous amount of phosphate from ATP, which could be preserved when the incubation was terminated by ice-cooled acetone approximately at maximum saturation which presented the intramolecular movements of phosphate groups. This myosin incorporates the phosphate from a relatively low ATP concentration compared with cerebellar and brain stem ones.

Alkaline hydrolysis releases only an insignificant amount of phosphate from N–P bonds. A larger amount of inorganic phosphate can be released from the ester-type bonds.

Making a comparison between the unphosphorylated and phosphorylated HBMs a significant variance is seen in the phosphate incorporation depending on the sources of preparations. While the cerebral KCl-myosin incorporates the phosphate into the P–Arg in the greatest proportion (see Table IV), the cerebellar KCl-myosin incorporates it into P–Arg, P–His and into the unidentified samples Nos 1, 2 and 9, while the brain stem one into P–Arg, P–His and compound No. 6.

The results support the existence of individual myosins in the CNS and the possibility of further myosin varieties in nervous tissues. Nevertheless,

it must be considered that all the myosins are the final products of a given procedure and, therefore, the real internal P content and composition of myosin is not reflected. It is thus suggested that the term "preparative myosin" be used [10].

We think finally that data shown in Figs 8 and 9 referring to the higher ATP concentration and the longer incubation time confirm the movements of phosphate groups in the HBMs and also the existence of the phosphate burst.

On the basis of these results it may be supposed that the cytokinetic movements in brain (neural) cells are performed at the cost of the high-energy-potential amino acid phosphates. Therefore, it is important that P movement takes place rather rapidly and it is an inherent characteristic with brain myosins depending on their sources; this fast mechanism coincides with the working process of brain cells. We assume this to be one of the principal mechanisms of internal movements.

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EFFECT OF INDOMETHACIN ON RENAL FUNCTION DURING DIFFERENT LEVELS OF SURGICAL STRESS

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To determine whether renal prostaglandins participate in the regulation of renal blood flow, sodium and water excretion during "stress situation", renal function was investigated in two groups of anaesthetized dogs, subjected to minor and to more severe surgical stress under control conditions, and following the administration of 4 mg/kg indomethacin i.v.

In the control studies, the renal haemodynamic parameters (C_{PAH}, C_{inulin}), urine output and sodium excretion were not different in those animals in which the surgical traumatization was more severe from data obtained in similarly anaesthetized dogs.

Extracellular volume expansion induced with i.v. infusion of Ringer solution enhanced sodium and water excretion in both groups, however, the increase of sodium excretion was less in the dogs subjected to more severe stress.

During indomethacin infusion glomerular filtration did not change in either groups; C_{PAH} decreased by 20–25% in the anaesthetized animals and 35–40% in dogs in which the surgical stress was more severe. In this group the total renal blood flow was reduced by 40% simultaneously with the haemodynamic changes; sodium and water excretion fell in both groups.

After indomethacin infusion the diuretic response of the kidneys to extracellular volume expansion was markedly reduced in the anaesthetized dogs, the diuretic and natriuretic effects being almost completely inhibited in the animals subjected to more severe stress.

These data suggest that in the anaesthetized dog endogenous prostaglandins may serve to maintain renal blood flow but not the glomerular filtration rate. Inhibition of prostaglandin synthesis during more severe stress results in increased renal vascular resistance and reduced renal blood flow. Accordingly, the data provide evidence that renal prostaglandins counteract in the kidney the vasoconstrictor mechanisms activated during more severe surgical traumatization.

The data do not support the direct physiological role of prostaglandins in regulating tubular function.

Data from several laboratories have recently demonstrated that the regulation of renal blood flow is based on a complex interplay of vasoconstrictor and vasodilator factors. In particular, prostaglandins are known to be involved in renal blood flow control in a variety of conditions in which the renin-angiotensin or the sympathetic nervous system is activated.

Although inhibition of prostaglandin synthesis has little or no effect upon renal blood flow in conscious, chronically instrumented dogs [20, 21], it may elicit or augment the reduction of renal blood flow that occurs in the anaesthetized animal.

These divergent results can be explained as follows. Renal vasoconstrictors such as epinephrine or norepinephrine [16], angiotensin I [15], angiotensin

II [14], renal nerve stimulation [17], and renal ischaemia [7, 14] increase renal prostaglandin release. Activation of any of these stimulatory pathways during an experimental procedure would be expected to potentiate the inhibitory actions of prostaglandin synthetase inhibitors by augmenting the level of renal synthesis of prostaglandins. If prostaglandin synthesis is inhibited, the influence of vasoconstrictors is accentuated.

Whereas conscious dogs increase renal sodium excretion after inhibition of prostaglandin synthetase [8], we have shown that indomethacin, a potent inhibitor of prostaglandin synthetase, decreases sodium and water excretion in anaesthetized dogs [9, 10]. The antidiuretic and antinatriuretic effect of indomethacin is probably secondary to renal vasoconstriction.

The purpose of the present study was to determine whether the surgical stress per se affected the kidney function during prostaglandin synthesis inhibition or not.

The experiments were carried out in two groups of anaesthetized dogs in which the degree of surgical stress was different. In the first series surgical stress was less while in the second one surgical traumatization was more severe. Simultaneous measurements of the renal parameters were performed under control conditions and following the administration of the inhibitor of prostaglandin synthesis.

Methods

The experiments were performed on mongrel dogs of either sex weighing 12 to 25 kg. For 24 hours prior to the experiment the animals were kept on water only. Anaesthesia was induced with sodium pentobarbital (25 mg/kg i.v.) and maintained with repeated additional doses. After the anaesthesia each animal received an infusion of Ringer solution equal to 1% of the body weight containing para-aminohippuric acid (PAH) and inulin ensuring a plasma concentration of 2 mg and 30 mg per 100 ml, respectively, followed by a sustaining infusion of 0.25 ml/kg per min for the rest of the experiment.

Both femoral arteries and veins were cannulated in all animals, for arterial blood collections and infusions. Mean arterial blood pressure was measured by a Statham strain-gauge transducer connected to a polyethylene catheter in the left femoral artery and recorded on a RADELKIS recorder.

From a low abdominal midline incision the bladder was exposed and the ureters were catheterized supravesically using fine polyethylene cannulas.

In another series of experiments, the left renal hilum was dissected through a left flank incision and retroperitoneal dissection, following cannulation of the ureters, femoral arteries and veins.

The left renal vein was connected to the left external jugular vein with a siliconized rubber tube. A T-extension of the tube permitted direct measurement of renal venous outflow. Prior to establishment of the anastomosis, 0.1 ml (500 I.U.) per kg body weight of heparin was given intravenously.

In this group, termed *cannulated group* the surgical stress was more severe than in the animals without flank incision, termed *non-cannulated* group.

After starting the infusion, 60 min were left for attaining an equilibrium, then urine was separately collected from the kidneys in 20-min periods. Blood was withdrawn at the midpoint of each clearance period. After centrifugation of the blood samples the supernatant plasma was kept for analysis and the erythrocytes were reinfused in Ringer solution. In the *cannulated group* renal blood flow was estimated twice in the middle of each period by measuring venous outflow (RBF_{dir}) indicated by the time necessary for 25 ml blood to be collected.

In the present paper we demonstrate the results of 4 experimental series. In Groups 1 and 2 (*non-cannulated* and *cannulated* dogs) the indomethacin solvent (50 ml 0.1 M phosphate-buffer diluted 4-fold with Ringer solution, pH 8.0) was infused i.v. for 10 min after the control period (Period 1).

The effects of the solvent on kidney function were examined during the second period (Period 2).

In these experiments the effect of a larger volume expansion was also studied on kidney function. Ringer solution, 25 ml per kg body weight prewarmed to body temperature, was infused for 60 min (Periods 3, 4, 5), and at the end of hydration, volume expansion was assumed to be 5% of body weight. Following volume expansion 3 postexpansion 20-min urine collection periods were made (Periods 6, 7, 8).

In Groups Nos 3 and 4 (*non-cannulated* and *cannulated* dogs) 4 mg/kg indomethacin dissolved in 50 ml of 4-fold diluted 0.1 M phosphate-buffer solution was infused i.v. for 10 min after the control period (Period 1); otherwise the experimental protocol was the same as previously described.

PAH concentration in the urine and plasma was determined by the method of SMITH et al. [19], that of inulin by the method of LITTLE [13]. Urinary sodium concentration was measured by flame photometry. Osmolality of the urine was measured by the method of freezing point depression using an *Advance osmometer*.

Haematocrit was determined by means of a *Hawksley* microhaematocrit centrifuge, plasma protein concentration by the biuret method.

The clearance of PAH (C_{PAH}) and the clearance of inulin (C_{inulin}) were determined by the usual formulas. All data were referred to 100 g kidney tissue with the exception of total renal vascular resistance, which was calculated per kg of kidney tissue.

Statistical significance of the data was determined by paired and unpaired analysis, using Student's t test. A p value of less than 0.05 was considered significant.

Results

Figure 1 shows that in the control animals (*non-cannulated group*, 13 animals; *cannulated group*, 10 animals) there was no significant change in arterial blood pressure following the administration of the indomethacin solvent.

On the figures only the parameters of the left *cannulated* kidney will be presented since mean values for all parameters in the right kidney were the same as in the left one, the magnitude of changes being also the same. For the sake of comparison variables referring to the right kidney will be given in the text.

The C_{PAH} in the first period in the *non-cannulated* dogs was 237 ± 13 ml/min, and in the left kidney of the *cannulated* dogs 260 ± 24 ml/min, while in the right intact kidney it attained 261 ± 22 ml/min; at the end of the hydration (Period 5) corresponding figures were 216 ± 16 ml/min, 233 ± 12 and $237 \pm \pm 11$ ml/min, respectively.

Mean value for C_{inulin} failed to change in the course of the experiment and was the same in both series. It is reasonable to assume that during extracellular volume expansion the glomerular filtration rate (GFR) remained constant. Mean value for C_{inulin} at the end of hydration (Period 5) in the *non-cannulated* animals was 81 ± 8 ml/min and 86 ± 8 ml/min in the left kidney of the *cannulated* group.

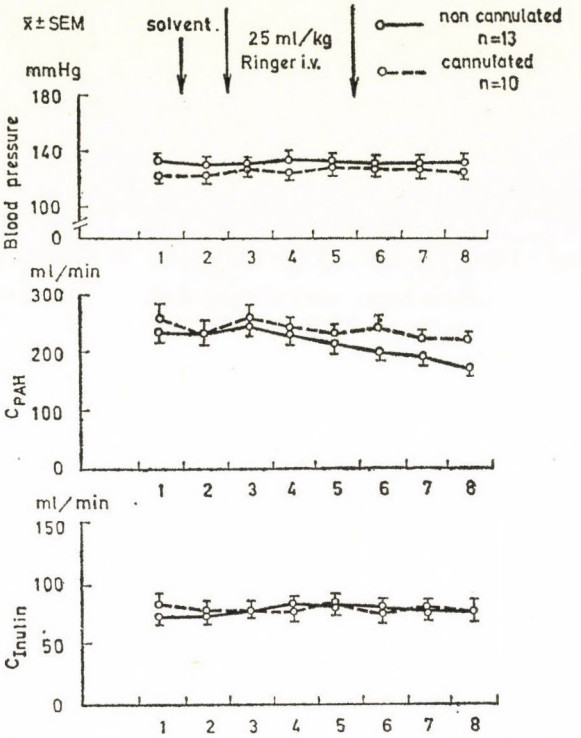

Fig. 1. Mean arterial pressure, clearance of PAH (C_{PAH}) and inulin (C_{inulin}) in the control animals (*non-cannulated* group and *cannulated* group). On the abscissa the number of each period is given (time of the periods was 20 min). Results are given as mean ±SEM

Urine output (Fig. 2) was 2.15±0.40 ml/min in the *non-cannulated* and 2.03±0.45 ml/min in the *cannulated* animals at the start of the experiments. The solvent of the indomethacin did not affect urine excretion.

During hydration urine output increased markedly. At the end of hydration (Period 5) urine volume was 6.57±0.73 ml/min in Group 1, and 5.40± ±0.54 ml/min in the left kidney of Group 2, while 5.40±0.40 ml/min in the right, intact kidney.

Sodium excretion during the control periods was 249±54 μEq/min in Group 1, 223±47 μEq/min and 212±52 μEq/min in Group 2. Hydration caused a steady increase in sodium output from 249±54 μEq/min to 405±77 μEq/min in the *non-cannulated* animals ($p < 0.001$), and did not change in the *cannulated* group. Urine osmolality decreased in both groups and during the extracellular hypervolaemia the urine became hypoosmotic as compared to the plasma (216±32 mosm/l, 174±10 mosm/l and 194±20 mosm/l).

Osmolality of the plasma was 315±3 mosm/l. There was no significant change in plasma osmolality in any group of experiments.

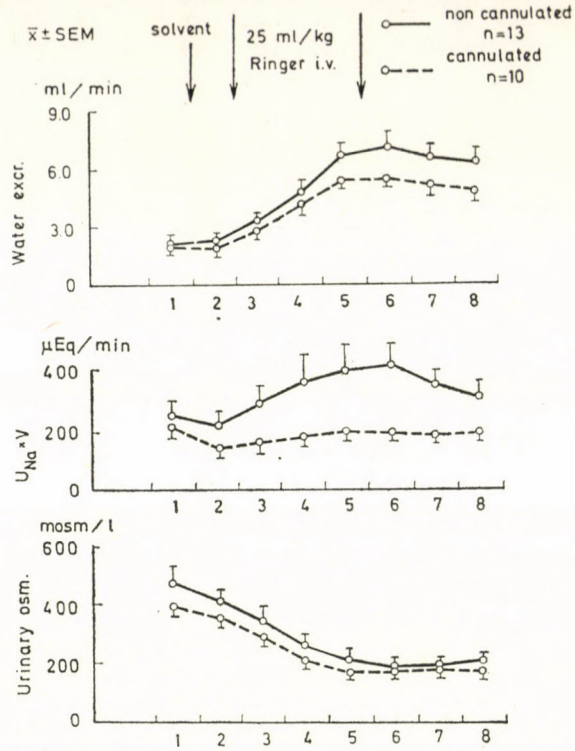

Fig. 2. Urine output, sodium excretion, urine osmolality in the *non-cannulated* and *cannulated* control animals

Figure 3 represents the data obtained after indomethacin infusion.

Indomethacin, 4 mg/kg i.v., caused a rise in arterial blood pressure in both groups.

Mean value for C_{PAH} was 235 ± 17 ml/min in the *non-cannulated* animals (Group 3) and 238 ± 18 ml/min in the *cannulated* dogs (Group 4) at the start of the experiment (Period 1). Following indomethacin infusion, C_{PAH} decreased to 216 ± 17 ml/min in Group 3 ($p < 0.01$ as compared with the control value) and to 145 ± 19 ml/min in Group 4, 132 ± 22 ml/min being recorded in the right kidney ($p < 0.001$ as compared with the control value).

The i.v. infusion of indomethacin resulted in a greater drop in C_{PAH} in the *cannulated* animals than in *non-cannulated* dogs during Period 5 ($p < 0.01$).

The GFR was 82 ± 7 ml/min in Group 3, 84 ± 10 ml/min in Group 4, and it failed to show any significant change.

Following indomethacin infusion urine output decreased in both groups (Fig. 4), in the *non-cannulated* dogs from 2.34 ± 0.60 ml/min to 1.49 ± 0.40 ml/min ($p < 0.001$) and in the *cannulated* animals from 1.71 ± 0.36 ml/min to

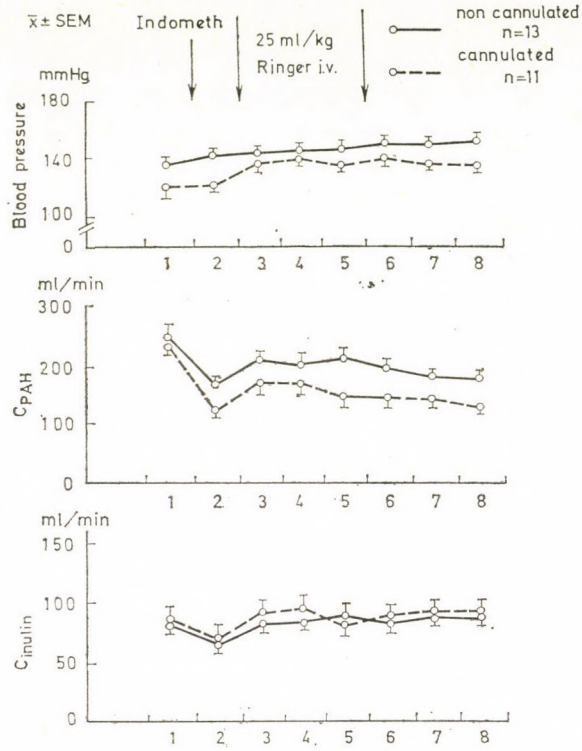

Fig. 3. Mean arterial blood pressure, C_{PAH}, C_{inulin} in the *non-cannulated* and *cannulated*, indomethacin treated dogs

0.60±0.13 ml/min in the left, and from 1.98±0.36 ml/min to 0.49±0.12 ml/min in the right kidney ($p < 0.01$).

In Group 3 urine excretion increased to 4.21±0.90 ml/min during hydration, however, this increase was smaller than that observed in the control kidneys. At the end of hydration (Period 5), urine output in Group 4 was 1.36±0.44 ml/min and 1.26±0.43 ml/min in the left and right kidney, respectively, indicating that in these animals indomethacin inhibited the diuretic effect of extracellular volume expansion.

After indomethacin infusion, sodium excretion fell from 282±95 μEq/min to 236±80 μEq/min ($p < 0.001$) in the *non-cannulated* animals, from 190±±53 μEq/min to 55±26 μEq/min ($p < 0.001$) in the *cannulated* group.

During perfusion with Ringer solution sodium excretion rose steadily in the *non-cannulated* group to 371±100 μEq/min in Period 5, while remained constant in the *cannulated* kidneys.

The infusion of indomethacin inhibited the natriuretic effect of extracellular volume expansion.

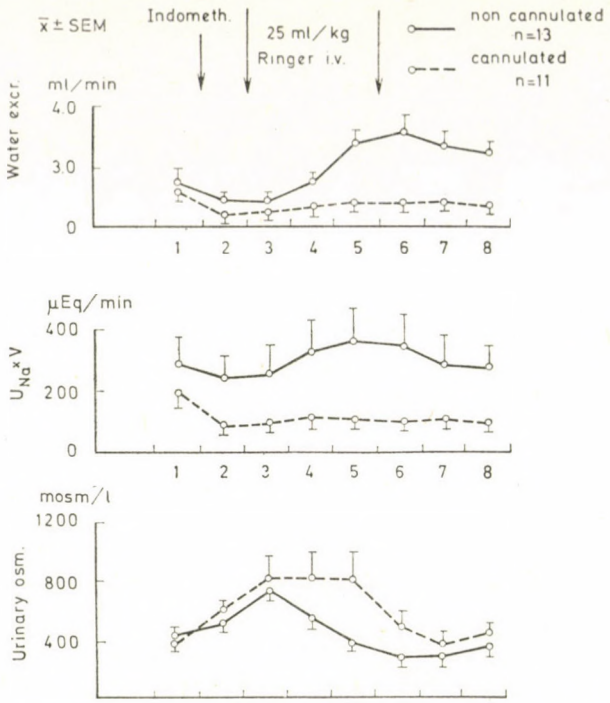

Fig. 4. Urine output, sodium excretion, urine osmolality in the control period and following the infusion of 4 mg/kg indomethacin i.v. in the *non-cannulated* and *cannulated* animals

Urine osmolality changed according to urine and sodium output, its value remaining higher than plasma osmolality.

Figure 5 represents the haemodynamic parameters of the *cannulated* kidneys. In the control animals the total vascular resistance of the kidney ($R_{kidney/kg}$) showed little change throughout the study. Following the infusion of indomethacin it increased from 1.16 ± 0.08 to 2.46 ± 0.24, then rose steadily attaining 3.41 ± 0.39 at the end of the experiment (Period 8).

Renal blood flow (RBF_{dir}) was 528 ± 25 ml/min in Group 3, and remained constant. Administration of indomethacin (Group 4) decreased RBF_{dir} from 510 ± 21 ml/min to 332 ± 34 ml/min ($p < 0.001$) a value of 274 ± 38 ml/min having been attained at the end of the study.

Haematocrit was $38 \pm 1.2\%$ in the untreated control dogs and $34 \pm \pm 0.9\%$ in those treated with indomethacin; these values showed little change throughout the study.

The plasma protein concentration was the same in the two series at the beginning of the experiment, 4.96 ± 0.19 g/100 ml and 4.80 ± 0.10 g/100 ml, being decreased during hydration in both groups to 3.84 ± 0.16 g/100 ml and 4.10 ± 0.10 g/100 ml, respectively.

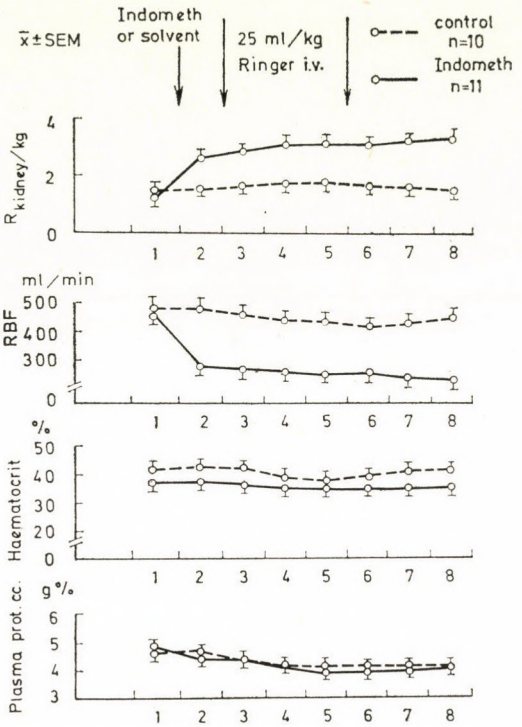

Fig. 5. Renal haemodynamic parameters in the *cannulated* animals. The solid lines represent data obtained in the control animals, the dotted lines show those of the indomethacin treated group. Data points represent mean ± SEM

Discussion

In the present work it has been shown that the antidiuretic and antinatriuretic effect of indomethacin is considerably increased in those animals in which surgical traumatization was more severe (*cannulated* animals).

The values for C_{PAH} were not significantly different in the control animals (Fig. 1), and no difference was measured in GFR between the *non-cannulated* and *cannulated* groups, which means that renal blood flow was maintained during the more severe stress. These results are in agreement with the data obtained by others in anaesthetized dogs [3, 12]. The absence of renal vasoconstriction during the acute reduction of cardiac output is noteworthy since arterial plasma renin activity and arterial plasma concentration of norepinephrine have been found to be elevated in response to thoracic caval constriction and because angiotensin II and norepinephrine are both potent renal vasoconstrictors [18].

Since prostaglandins have intrinsic renal vasodilator action [14], it is likely that they might attenuate the renal vascular effects of norepinephrin and angiotensin II by directly acting upon the vascular smooth muscle cells. Support for this interpretation may be derived from studies indicating that the renal vasoconstrictor effects of exogenous angiotensin II [5], norepinephrine [11], and renal nerve stimulation [4] enhance the production of renal prostaglandins. Biosynthesis of PGE_2 has been demonstrated in vascular endothelial and smooth muscle cells [1, 6], as well as in renal interstitial cells [22]. The mechanism(s) whereby the prostaglandins attenuate renal vasoconstrictor processes remains to be determined.

In the present study C_{PAH} was lowered by 20–25% in the *non-cannulated* group and by 35–40% in the *cannulated* dogs following indomethacin infusion, and renal blood flos (RBF_{dir}) decreased by 40% in Group 4. Despite the significant decrease in renal plasma flow induced by inhibition of prostaglandin synthesis, glomerular filtration did not change in our experiments. This finding is in agreement with other studies in which inhibition of prostaglandins reduced renal blood flow but had no effect on glomerular filtration rate [9, 10]. The present study demonstrates that the vasoconstriction occuring in the kidney after the administration of indomethacin was augmented during the more severe stress in the kidney of the *cannulated* group.

These data suggest that the renal prostaglandins were counteracting the vasoconstrictor mechanisms activated during more severe surgical traumatization and that after inhibition of renal prostaglandin synthesis the effect of the vasoconstrictor mechanisms was more expressed on renal circulation.

As shown in Fig. 4, urine output and the sodium excreation decreased in both groups, after indomethacin infusion, however, the fall was more pronounced in the *cannulated* animals. In this latter group indomethacin almost completely inhibited the natriuretic and diuretic effects of isosmotic extracellular volume expansion.

The data of the present study are also consistent with results obtained in an other series of experiments in which the antagonism of furosemide and indomethacin was investigated [9].

The fact that water and sodium excretion decreased following indomethacin infusion in spite of unchanged GFR may be explained by an increased water and sodium reabsorption in the proximal tubules [9, 10]. Indomethacin may certainly influence the factors (haematocrit, peritubular capillary pressure, postglomerular plasma protein concentration, etc.) increasing proximal tubular sodium and water reabsorption, and therefore a smaller part of the glomerular filtrate is reaching the distal part of the nephron.

In our experiments indomethacin produced an increase in renal vascular resistance which was more marked in the *cannulated* group during the more severe stress, followed by a marked drop in urine and sodium excretion.

These results may be interpreted as follows. The larger fall in renal blood flow results in more marked changes in the Starling forces around the proximal convolution, resulting in a substantial increase of proximal tubular reabsorption and a decrease in urine and sodium excretion.

These data are consistent with the concept that intrarenal PG's play a role in the adjustment of renal vascular resistance, and support their role in the regulation of salt and water excretion. These results do not provide any evidence for a primary role of the PG-system in the regulation of tubular function.

In the dogs treated with indomethacin medullary blood flow was lower than in the control animals suggesting that the renal prostaglandins are participating in the regulation of renal medullary blood flow [2]. The decrease of medullary blood flow causes an increase of the medullary solute concentration, resulting in enhanced water reabsorption in the collecting ducts.

In our experiments no attempt was made to investigate the effects of indomethacin on medullary flow.

Theoretically, the renal medullary vasoconstriction that followed indomethacin infusion could have a supplementary intrarenal osmotic effect increasing water reabsorption and decreasing urine output.

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STUDY OF THE INDOMETHACIN AND BRADYKININ ANTAGONISM IN ANAESTHETIZED DOGS

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To determine the prostaglandin dependent and independent effects of bradykinin in the kidney, bradykinin (0.05 μg/min/kg body weight) was infused into the left renal artery during inhibition of prostaglandin synthesis by indomethacin.

Indomethacin, 0.1 mg/min/kg body weight i.v. produced a marked fall in urine output and sodium excretion in anaesthetized dogs. Renal vascular resistance increased and renal blood flow (RBF_{dir}) decreased by 30%.

When during the i.v. infusion of indomethacin bradykinin was infused into the left renal artery, RBF_{dir}, urine flow and sodium excretion increased to the control values in the left kidney while remained lower in the right kidney.

The results suggest that bradykinin increases renal blood flow by an action which does not require the mediation of prostaglandins.

A decrease in renal blood flow, which was a constant feature during i.v. indomethacin infusion, is probably responsible for the decrease in urine flow and sodium excretion.

The failure of indomethacin to inhibit the natriuretic and diuretic effects of bradykinin suggests that the prostaglandins are not important determinants of these responses.

Renal arterial infusion of prostaglandins [13, 23] or stimulation of intrarenal synthesis of prostaglandins by renal arterial infusion of arachidonic acid [29] increase renal blood flow and the excretion of sodium chloride and water. There is evidence to suggest that prostaglandins (PG's) synthetized and liberated in the kidney function as local hormones participating in the regulation of renal blood flow and its intrarenal distribution [4, 24, 16].

Treatment with indomethacin, a potent inhibitor of renal PG biosynthesis *in vitro* [6] and *in vivo* [20], reduces renal blood flow [11, 18], affecting similarly urine and sodium excretion [9, 18].

The vasodilator and natriuretic effects of bradykinin in the kidney are well established [17]. Renal arterial infusion of bradykinin increases prostaglandins in the renal venous blood and in urine [8, 12], presumably by activation of a phospholipase A_2 [15].

The similar effects of bradykinin and prostaglandins on renal function and the ability of bradykinin to stimulate prostaglandin synthesis suggest that prostaglandins may mediate the actions of bradykinin in the kidney.

The present study was undertaken to investigate the effects of bradykinin on renal blood flow, urine output, and sodium excretion during prostaglandin synthesis inhibition, in an attempt to determine to what extent the bradykinin effects are mediated by prostaglandins.

Methods

The experiments were performed on mongrel dogs of either sex weighing between 20–30 kg. For 24 hours prior to the experiment the animals were kept on water only. Anaesthesia was induced with sodium pentobarbital (25 mg/kg i.v.) and maintained with periodic additional doses. After anaesthesia each animal received an infusion of Ringer solution at body temperature equal to 1% of the body weight, containing para-aminohippuric acid (PAH) and inulin ensuring a plasma concentration of 2 mg and 30 mg per 100 ml, respectively, followed by a sustaining infusion of 0.25 ml/kg/min for the remainder of the experiment.

Cannulation of the femoral arteries and veins on both sides were performed in all animals, for arterial blood collections and infusions. Mean arterial blood pressure was measured by a Statham strain-gauge transducer connected to a polyethylene catheter placed in the left femoral artery, and recorded on a RADELKIS recorder.

From a lower midline incision the bladder was exposed and the ureters were catheterized supravesically using fine polyethylene cannulas.

Through a left flank incision and retroperitoneal dissection, the left renal hilum was exposed. The left renal vein was connected to the left external jugular vein with a siliconized rubber tube. A T-extension of the tube permitted the direct measurement of renal venous outflow. Prior to anastomosis 0.1 ml (500 I.U.)/kg body weight of heparin was given intravenously.

After starting the infusion, 60 min were allowed for equilibrium, the urine was separately collected from the kidneys in 2 successive 10-min periods. Blood was taken at the midpoint of each clearance period. In all instances arterial and renal vein blood samples were obtained simultaneously. After centrifugation, the supernatant plasma was removed and the erythrocytes were reinfused in Ringer solution.

In the middle of each clearance period renal blood flow was estimated twice from the venous outflow (RBF_{dir}) by measuring the time necessary for an output of 25 ml blood. Values obtained in these two periods served as controls.

Following the two control periods, indomethacin 0.1 mg/kg body weight/min, dissolved in 1 ml 4-fold diluted 0.1 M phosphate-buffer solution (pH 8.0) was infused i.v.

Ten min were left for equilibrium, then the effects of indomethacin on renal function were studied in two successive 10-min periods. Subsequently to the i.v. constant indomethacin infusion 0.05 μg/kg/min of bradykinin (SANDOZ) was infused directly into the left renal artery in a volume 0.5 ml/min physiological saline.

Five minutes later variables of renal function were again determined in 2 successive 10-min periods.

PAH concentration in urine and plasma was determined by the method of SMITH et al. [26], that of inulin by the method of LITTLE [21]. The urinary sodium concentration was measured by flame photometry. Haematocrit was determined by means of Hawksley microhaematocrit centrifuge.

The clearance of PAH (C_{PAH}), clearance of inulin (C_{inulin}), the extraction of PAH (E_{PAH}) and inulin (E_{inulin}) were determined by the usual formulas.

All data were referred to 100 g kidney tissue with the exception of total renal vascular resistance which was calculated per kg kidney tissue, using the equation

$$R_{kidney/kg} = \frac{\text{Arterial blood pressure (mm Hg)}}{RBF_{dir}(\text{ml}) \times \text{kg}^{-1}\ \text{kidney weight} \times \text{sec}^{-1}}.$$

For mathematical analysis, Student's paired *t*-test was used.

Results

Changes observed in the parameters of the left kidney in 10 dogs are presented in Fig. 1. All values are mean ±SD. In each animal the average of the two clearance periods was used for calculation of the mean of the group.

During indomethacin infusion mean arterial pressure increased steadily from 124±10 mm Hg to 135±11 mm Hg, and then to 142±10 mm Hg.

The intravenous indomethacin infusion resulted in a significant increase of renal vascular resistance ($R_{kidney/kg}$) from 1.61±0.26 in the control period to 2.69±1.03 during indomethacin infusion. When during the i.v. infusion of indomethacin bradykinin was infused into the left renal artery $R_{kidney/kg}$ decreased to 2.00±0.69. RBF_{dir} decreased from 476±89 ml/min to 337±99 ml/min during indomethacin infusion, and increased to 451±99 ml/min under the joint effect of indomethacin and bradykinin.

During indomethacin infusion C_{PAH} decreased in the left kidney from 211±44 ml/min to 108±40 ml/min and in the right kidney from 222±29 ml/min to 115±35 ml/min. Comparing the indomethacin periods to the values

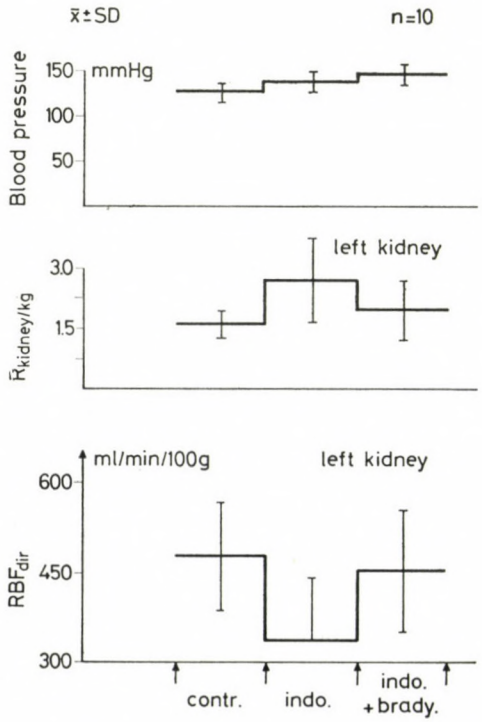

Fig. 1. Effect of treatment with indomethacin on the response to infusion of bradykinin into the left renal artery in blood pressure, total renal vascular resistance ($R_{kidney/kg}$), and renal blood flow (RBF_{dir}) in the left kidney

obtained during indomethacin plus bradykinin, C_{PAH} increased in the left kidney to 200±110 ml/min while remained lower in the right kidney at 104± ±54 ml/min (Fig. 2). Under the effect of indomethacin C_{inulin} was lowered in the left kidney from 77±11 ml/min to 51±18 ml/min while in the right one from 80±27 ml/min to 56±12 ml/min. The bradykinin infusion into the left renal artery augmented the gomerular filtration rate in the left kidney to 75±27 ml/min while did not influence it in the right kidney (60±35 ml/min).

In the left kidney infusion of indomethacin increased E_{PAH} significantly from 0.72±0.09 to 0.82±0.08 ($p < 0.001$), E_{inulin} from 0.27±0.05 to 0.36± ±0.08 ($p < 0.001$) while bradykinin infusion lowered both values (E_{PAH} to 0.70±0.14, and E_{inulin} to 0.32±0.08).

Indomethacin administration resulted in a considerable drop of urine output and sodium excretion (Fig. 3). In the left kidney urine output decreased from 2.83±1.97 ml/min to 0.80±0.76 ml/min and in the right kidney from 2.47±1.27 ml/min to 0.52±0.34 ml/min. During the infusion of bradykinin,

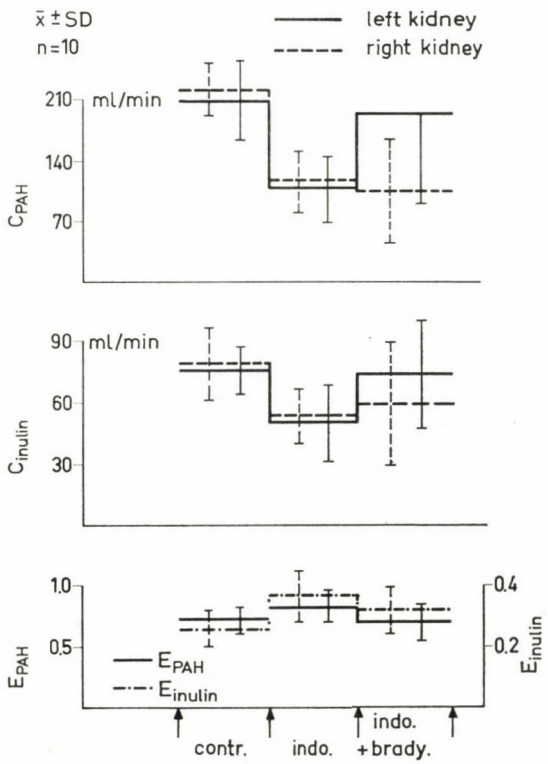

Fig. 2. Effect of treatment with indomethacin on the response to infusion of bradykinin into the left renal artery in C_{PAH}, C_{inulin} in the left kidney (solid line) and in the right kidney (broken line), and E_{PAH}, E_{inulin} in the left kidney

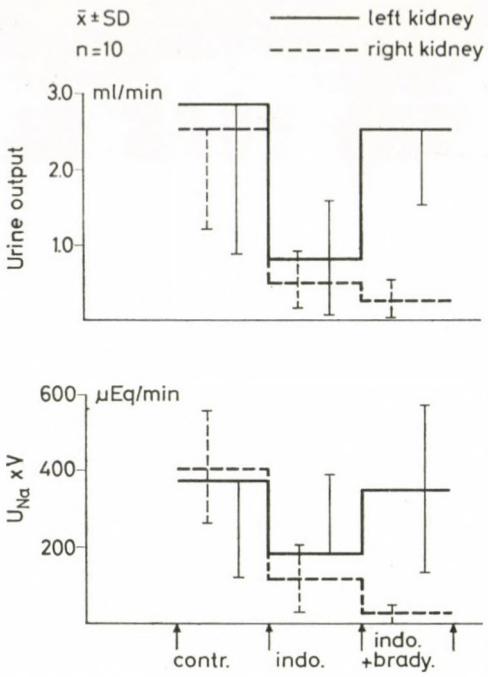

Fig. 3. Effects of treatment with indomethacin on the response to infusion of bradykinin into the left renal artery in urine flow and sodium excretion in the left kidney (solid line) and in the right kidney (broken line)

urine volume increased in the left kidney to 2.50±0.95 ml/min and decreased in the right kidney to 0.22±0.21 ml/min.

Sodium excretion from the left kidney was 372±240 μEq/min and from the right kidney it was 409±132 μEq/min during the control periods, while these values decreased following indomethacin infusion to 195±190 μEq/min and 119±88 μEq/min, respectively. Under the joint effect of indomethacin and bradykinin sodium excretion increased from the left kidney to 350±±223 μEq/min while it fell from the right kidney to 25±20 μEq/min.

Discussion

Results of the present study show in accordance with earlier findings [11, 16] that the infusion of indomethacin, an inhibitor of prostaglandin synthesis, decreases renal blood flow, urine flow rate, and sodium excretion.

The observation that indomethacin decreased renal blood flow is consistent with the findings of Lonigro et al. [22] and Aiken and Vane [1] who measured RBP with electromagnetic flowmeters in anaesthetized dogs. The

marked decrease in renal plasma flow in this study measured by PAH clearance, a technique which would not be expected to adversely affect the kidney or interfere with the renal nerves, is remarkably similar to the decrease reported by LONIGRO et al. [22] and supports the hypothesis that in the anaesthetized dog renal blood flow may be dependent on prostaglandin synthesis.

We have previously shown that indomethacin treatment does not affect glomerular filtration rate [18]. These findings agree with those of ANDERSON et al. [2] and suggest that maintenance of GFR is not dependent on prostaglandin synthesis. However, the present results show that the C_{inulin} may fall during indomethacin infusion.

The apparent conflict in these results may be resolved by considering the very low urine output (0.80 ± 0.76 ml/min) which might have caused in accuracies owing to the "dead space". The direct clearance of inulin ($RPF \times E_{inulin}$) remained constant, proving that GFR did not decrease under the effect of indomethacin in spite of the low RBF_{dir}.

Results of the present investigation demonstrate that 10 min after the administration of indomethacin there was a marked decrease in urine flow and sodium excretion. The results of the present study are consistent both with data of ATTALAH and LEE [3] and with our previous observations [19], suggesting that a single effect of prostaglandin synthesis inhibition is responsible for both changes. Renal vasoconstriction was indicated in our experiments by the reported increase in renal vascular resistance and decreased renal blood flow. In addition, filtration fraction (E_{inulin}) was markedly elevated. These two findings would be expected to decrease peritubular hydrostatic pressure and increase peritubular oncotic forces in the proximal tubules, which would lead to increased sodium and water reabsorption in this part of the nephron.

KIRSCHENBAUM et al. [16] have demonstrated a relative shift of intrarenal blood flow to outer cortical regions of the kidney during administration of indomethacin. It is possible that such a shift in blood flow resulted in increased PAH extraction (E_{PAH}) through passive means in the present experiments, too.

In detailed studies of renal blood flow by the radioactive microsphere technique it has been shown that the renal cortex can be divided into three areas of regional flow: [1] superficial nephrons, [2] mid-cortical nephrons, and [3] juxtamedullary nephrons [27]. Studies of the effect of bradykinin on the distribution of blood flow to these three regions showed that bradykinin increased flow to mid- and innercortical zones without a change in flow to the outer cortical zone [28]. In our previous study bradykinin infusion into the renal artery markedly increased renal blood flow and significantly decreased PAH extraction indicating that an increase in blood flow to innercortical or juxtamedullary nephrons is probably associated with an increase in medullary or non-cortical blood flow.

The response of renal plasma flow (RBF) to bradykinin during indomethacin infusion observed in the present study and assessed by the changes in E_{PAH}, namely an increase in cortical and non-cortical plasma flow, represents a patterns of distribution of flow similar to that observed in the studies cited above.

The rise of renal blood flow after bradykinin infusion with indomethacin pretreatment suggest a non-prostaglandin mechanism.

Micropuncture studies indicate that bradykinin affects neither nephron filtration fraction [27] nor proximal tubular sodium reabsorption in superficial cortical nephrons [5, 25, 27], and these findings suggest that the observed decrease in whole kidney filtration fraction (E_{inulin}) must be limited to deeper cortical nephrons such as the mid-cortical and juxtamedullary ones.

The results of BROUHARD et al. [7] suggest that bradykinin increases cortical plasma flow by stimulation of prostaglandin synthesis, while non-cortical plasma flow and sodium excretion by an action which does not require the mediation of prostaglandins.

If prostaglandins participate in the renal vasodilator action of bradykinin, one might anticipate that prostaglandin synthesis inhibition would reduce the response to bradykinin. Since the response to bradykinin was not reduced, our data indicate that in the canine renal vascular bed the effects of bradykinin are probably not dependent on the release of vasodilator prostaglandins.

Injection of bradykinin into the artery produced similar vasodilation both before and after indomethacin, indicating that in the canine coronary vasculature bradykinin failed to release significant amounts of dilator prostaglandins, or also the released prostaglandins were not significantly involved in the vasodilator action of bradykinin [14].

Because of the large number of conflicting data concerning the role of prostaglandins in the vasodilator activity of bradykinin, it appears that the relationship between these vasoactive hormones has yet to be elucidated [10].

Based largely on our experimental data [18, 19], we have concluded that sodium chloride transport in the canine nephron is neither inhibited nor stimulated by the renal PG's. The antidiuretic and antinatriuretic effects of prostaglandin synthesis inhibition by indomethacin in our studies were considered to be to secondary to the decrease of renal blood flow.

The dogs treated with indomethacin responded to bradykinin with an increase in renal blood flow, urine and sodium excretion. The increase in renal blood flow that followed bradykinin infusion might have been responsible for the natriuresis and the enhancement of urine formation by the same mechanism as proposed above to explain the antidiuretic and antinatriuretic effects of indomethacin.

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THE EFFECT OF ANTIDIURETIC HORMONE ON INTRARENAL MICROSPHERE DISTRIBUTION

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The aim of the present study was to reexamine the effect of antidiuretic hormone on intrarenal blood flow distribution by the microsphere technique in anaesthetized rats. The administration of 3, 6 or 60 mU/kg/h of arginine-vasopressin (VP) was accompanied by a dose-dependent antidiuresis. Glomerular filtration rate increased only at the dose level of 60 mU/kg/h and was not significantly altered by the lower doses. Total renal blood flow was not significantly influenced by any of the doses of VP. The two small doses caused no change in intracortical microsphere distribution but there was a significant decrease in the ratio of outer to inner cortical blood flow when 60 mU/kg/h of VP was infused.

The results indicate that physiological doses of VP do not alter the distribution of intrarenal blood flow and show that the antidiuretic effect of VP is independent of changes in renal haemodynamics.

An unresolved controversy exists concerning the intrarenal haemodynamic effects of antidiuretic hormone (ADH). Since it is well accepted that the concentrating ability of the kidney is critically dependent on medullary blood flow it was hypothetized that the antidiuretic effect of ADH might, in part, be mediated by a reduction in medullary blood flow [13, 24]. However, when this hypothesis was tested experimentally, contradictory results were obtained. Studies claiming redistribution of blood flow toward the superficial cortex [4, 6, 13, 21, 24] or toward the deep cortex or medulla [1, 12, 15, 16] as well as no redistribution at all [2, 9, 11, 22, 25] have been reported following the administration of ADH. Such diversity of the results could not be explained by the diversity of the methods used to measure intrarenal haemodynamics since studies utilizing similar or even the same method, e.g. the microsphere method, are conflicting [1, 4, 9, 15, 16]. There were, however, great differences between the various studies in the state of hydration and, consequently, in endogenous ADH levels and furthermore, doses of exogenous ADH ranging from 1.5 to 96 mU/kg/h have been employed [1, 2, 4, 9, 11, 15, 16, 22]. In order to overcome these difficulties, in the present study the dose-response relationship between ADH and intracortical microsphere distribution was investigated during steady-state water diuresis in rats.

Materials and Methods

Male CFY rats (240–300 g) maintained on a standard laboratory chow were anaesthetized with Inactin® 100 mg/kg body weight, i.p. and tracheotomized. The left jugular vein was cannulated with a PE-50 tubing and an infusion of 77 mM/l NaCl solution containing ^{3}H-methoxyinulin was started at a rate of 1 ml/min/kg. A stretched PE-50 tubing was placed into the left femoral artery and a PE−10 tubing was inserted into the right femoral artery and advanced into the thoracic aorta. Both ureters were cannulated with unstretched PE-10 tubing. Whe urine flow rate had stabilized, usually two hours after starting the intravenous infusion, a control clearance period was obtained. Radioactive microspheres tagged with ^{85}Sr or ^{141}Ce (15 ± 1.5 μ, 3 M Co.) were then administered into the thoracic aorta. 12 000–15 000 microspheres (suspended in 0.4 ml of 154 mM NaCl containing 0.02% Tween-80) were injected over a period of 15–20 sec. Simultaneously, a reference blood sample was obtained from the left femoral artery at a rate of 0.4 ml/min. Blood withdrawal was started several seconds before the start of the injection and was continued for 10 sec after finishing it. After this a second control urine collection was made. This was followed by the administration of VP (Synthetic arginine vasopressin, grade VI, Sigma; 3, 6 and 60 mU/kg/h, respectively) for 30 min. A second bolus of microspheres was injected 20 min after starting the VP infusion. Two urine collections were made during the infusion of VP, one preceding and one following the microsphere injection. All urine collection periods lasted 10 min and arterial blood samples were obtained before and after each collection period. Mean arterial pressure was monitored with a Statham 23 dB strain-gauge.

At the termination of the experiments the kidneys were excised, weighed, and fixed in 10% formaldehyde. On the next day the renal cortex was divided into outer and inner zones. Gamma radioactivity of the samples was determined by a Beckman Biogamma Spectrometer and local blood flow was calculated using the following formula [20]:

$$\frac{\text{local blood flow}}{\text{activity in the sample}} = \frac{\text{rate of reference flow}}{\text{activity in reference blood}} .$$

^{3}H-radioactivity of the plasma and urine samples was determined by a Beckman LS-250 liquid scintillation counter and osmotic concentration by freezing point depression. Values obtained in the two control and two experimental periods were averaged. The data were statistically evaluated by Student's t-test for paired values.

Results

VP-induced changes in urinary osmotic concentration and in renal haemodynamics are summarized in Table I. As expected, VP administration was accompanied by a significant, dose-dependent antidiuresis. The three different doses of VP (3, 6 and 60 mU/kg/h) elevated urine osmolality by 136, 219 and 412%, respectively. Mean arterial pressure was not significantly influenced by 3 mU/kg/h of VP but with a dose of 6 mU/kg/h there was a small but statistically significant increase in this parameter and 60 mU/kg/h of VP increased mean arterial pressure by 12 mm Hg. This dose of VP caused a 24% increase in GFR. Total renal blood flow was not significantly influenced by any of the doses of VP. There was a tendency for filtration fraction to increase but this change did not reach statistical significance. The two lower doses of VP which are comparable with endogenous secretion rates during moderate water deprivation and can thus be regarded as physiological doses, caused no

Table I

The effect of arginine vasopressin on urinary osmotic concentration and renal haemodynamics in the rat

	V ml/min	U_{osmol} μosmol/ml	MAP mmHg	GFR ml/min	RBF ml/min	FF	OC/IC
	3 mU/kg/h	3 mU/kg/h VP,	n=9				
Control	0.102±0.010	83±4	120±5	0.85±0.08	5.86±1.02	0.27±0.035	5.11±1.19
VP	0.082±0.014*	196±29***	122±5	0.87±0.08	5.64±0.41	0.27±0.039	5.03±0.81
	6 mU/kg/h VP	6 mU/kg/h VP,	n=8				
Control	0.116±0.012	89± 7	122±4	0.86±0.05	5.73±1.14	0.27±0.04	5.07±0.91
VP	0.074±0.013*	294±30***	127±3*	0.90±0.06	5.62±0.61	0.28±0.02	4.29±0.68
	60 mU/kg/h	60 mU/kg/h VP,	n=9				
Control	0.109±0.008	87± 8	122±4	0.91±0.05	5.84±1.10	0.28±0.06	4.97±0.87
VP	0.069±0.006**	445±18***	134±5**	1.13±0.03**	5.71±0.90	0.33±0.05	3.74±0.59***

Values are means ±SE. V, urine flow rate; U_{osmol}, urinary osmotic concentration; MAP, mean arterial pressure; GFR, glomerular filtration rate; RBF, total renal blood flow; FF, filtration fraction; OC/IC, ratio of outer to inner cortical blood flow; V, GFR and RBF are referred to 1 g kidney weight. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

change in intracortical microsphere distribution. The dose of 60 mU/kg/h, however, elicited a small but statistically significant decrease in the ratio of outer to inner cortical blood flow. No correlation could be detected between VP-induced changes in urine osmolality and changes in renal haemodynamics.

Discussion

The values reported for the biological half-life of ADH in the rat are in the range of 1.0 to 3.7 min [17]. The volume distribution of VP is 25–50 ml/kg [7, 8] and its concentration in the blood after 24 h of water deprivation is 15–20 μU/ml [8, 19]. Thus, the calculated VP secretion rate in mild hydropenia is between 50–500 μU/kg/min. This calculated secretion rate is comparable to the two small doses of VP used in this study and can be regarded as "physiologic" if it is assumed that endogenous VP secretion rates were adequately suppressed. The assumption, however, seems to be justified even without blood VP determinations since all animals had control urine osmolalities below 100 mosmol/kgH_2O which indicates a negligible endogenous secretion.

The present data clearly show that physiological doses of VP have no measurable effect on intrarenal microsphere distribution, while after a pharmacological amount of VP intracortical blood flow distribution is shifted toward the inner cortex. Although in previous studies dose response relationships have not been reported the present results agree with most previous reports in that they showed no changes of intrarenal haemodynamics following the infusion of low or moderate doses of VP [2, 9, 11, 25] and an increase in inner cortical or outer medullary perfusion following treatment with higher doses [15, 16]. Several reports have claimed a VP-induced reduction in medullary flow [13, 14, 21, 24]. These results are, however, difficult to interpret in view of the criticism that has been advanced against the methods used to measure medullary flow [5]. The results of BANKS [4] are, however, not so easy to dismiss. He found that in rats and dogs with diabetes insipidus VP caused a decrease in the ratio of inner to outer cortical microsphere distribution. The reason for the contradiction with the present results is not readily apparent. One possibility is that diabetes insipidus in itself leads to an altered VP responsiveness. It is also possible that the controversy may be due to difficulties related to the microsphere technique [3]. Furthermore, it should be noted that VP may cause complex changes in the activity of the local hormone systems of the kidney, for instance, it has been shown to increase renal prostaglandin synthesis [18, 27] and to increase kallikrein secretion [10], and it is known to decrease renin secretion [23, 26]. These complex changes in the activity of vasoactive renal hormones may indirectly cause intrarenal haemodynamic effects which may be different from the direct effect of VP.

In summary, the present results failed to support the concept that physiological doses of VP alter the distribution of intrarenal blood flow, and they indicate that the antidiuretic effect of VP is independent of the changes of renal haemodynamics.

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PHARMACOLOGIC ASSESSMENT OF THE FUNCTIONAL STATE IN STENOSED CORONARY CIRCULATION OF THE DOG

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In open chest dogs under sodium pentobarbital anaesthesia the interaction of mechanical constriction on a large coronary branch and autoregulatory capacity of the relevant small resistance vessels was analyzed. Coronary blood flow (CBF) was measured with an electromagnetic flowmeter. Step-by-step mechanical constriction gradually abolished adenosine-induced coronary vasodilation, whereas the resting level of mean CBF remained unaltered. At this point verapamil (0.2 mg/kg i.v.), a vasodilator with a strong potency of blocking adenosine action, eventually decreased CBF and increased coronary resistance. Similar results were obtained with these drugs injected directly into a bypass established between the carotid and left common coronary arteries. The results suggest that (i) adenosine affects the same coronary segments which accomplish compensatory autoregulation (ii); with critical stenosis verapamil augments indirectly coronary resistance by inhibiting an "intrinsic" adenosine effect (iii); the functional state of stenosed coronaries can be assessed with the aid of these pharmacologic tests.

When myocardial blood supply is compromised by coronary stenosis resting level of blood flow may remain unaltered still the consequences of mechanical constriction exhaust metabolic autoregulatory capacity [3]. In fact, small vessel reactions mask the effect of stenosis on flow. It should be of considerable interest to know whether this interplay could be unmasked by pharmacologic means. With this in mind, we have examined the effect of verapamil since according to our previous studies [4] this drug exerts a highly selective depressant action on metabolic coronary autoregulation.

Methods

Dogs weighing 14–23 kg were anaesthetized with pentobarbital (30 mg/kg) and maintained on artificial respiration. The chest was opened in the fourth intercostal space and the heart was suspended in a pericardiac cradle. Blood pressure was measured in the femoral artery with a Statham P23 Db gauge, whereas a Statham SP 2202 electromagnetic flowmeter was used for the determination of coronary blood flow. The dogs were grouped into two experimental series. In Series I (6 dogs) the flow of the left anterior descending (LAD) coronary artery was measured. To allow constriction of the artery a snare occluder with a special screw was placed distal to the flow probe. In Series II (6 dogs) the left common coronary artery was cannulated at its origin with an Eckstein cannula [2] introduced through the left subclavian artery. The inflow side of the cannula was connected by means of a 30 cm long thick-walled inelastic rubber tubing (o.d. 7 mm, i.d. 4 mm) to the cannulated stump of the left common carotid artery. In this series, the flow probe was positioned on the carotid artery proximal to the cannula. Mechanical constriction of the artificial circuit was effected by a screw clamp on

the rubber tubing. Effective coronary perfusion pressure was monitored with another Statham gauge distal to the clamp.

Studies were conducted as follows: In both series the ability of the coronary bed to vasodilate was tested with bolus injections of adenosine. In Series I this was made by injecting 250 μg of adenosine through a catheter advanced into the left ventricular cavity: in Series II 5–10 μg of adenosine was injected directly into the rubber tubing. Thereafter, arterial coronary inflow was restricted mechanically in a step-by-step way, while the adenosine effect was elicited at every step of the constriction. The criterion for sufficient constriction was the reduction of the adenosine-induced flow increase to less than 20% of its control value. This stage was arbitrarily considered the starting point of the verapamil-test: the drug was injected in a dose of 0.2 mg/kg i.v. (Series I) or in a dose of $\simeq$ 8 μg/100 ml/min coronary blood flow intracoronarially (Series II). Afterwards, the constriction was removed and the preparation was allowed to regain circulatory equilibrium for at least 90 min. At the ending point of this period adenosine sensitivity and verapamil-tests were performed on the unrestricted vessels with the same dose of drugs (normal control). In every second animal of each series the protocol was performed in the reversed sequence: verapamil was first tested in the normal state and it was repeated in the constricted state 90 min thereafter. Consequently, both types of protocol are represented by three dogs in each series, and the data obtained in the 6 dogs were pooled. In addition to the above experiments, 3 dogs were used to investigate the effect of i.v. verapamil on adenosine-induced coronary vasodilation (see Discussion). Student's *t*-test for paired data was utilized for examining verapamil effects. Normal and constricted states were compared by using the *t*-test for unpaired data. All values are mean $\pm$ S.E.M.

Results

Experimental series I (non-cannulated vessels, 6 dogs)

By constricting the LAD artery in a step-by-step way, drastic reductions of the coronary vasodilating capacity can be achieved without affecting mean blood flow and arterial pressure. Figure 1 illustrates the effect of mechanical constriction on the adenosine-induced increase of coronary flow and calculated vascular conductance. According to the experimental protocol described in Methods, the effect of the Ca antagonist verapamil was tested both in unrestricted (normal) and in constricted ("adenosine-resistant") states of the coronary circulation (Table I). The predominant feature of the results was the apparent

Table I

*Effect of verapamil (0.2 mg/kg i.v.) on the coronary circulation**

	Arterial blood pressure (kPa)***		Coronary blood flow** (ml/min)		Vascular conductance (%)	
	Control	Verapamil	Control	Verapamil	Control	Verapamil
A Normal vessels	17.0±1.2	14.6±0.7[a]	28.6±4.1	37.1±6.8[a]	100 ±0	146.5±7.3[a]
B Constricted vessels	16.8±1.6	13.3±1.2[a]	26.8±3.3	15.8±1.9[a]	98.0±4.7	76.1±11.2[a]
P A-B	N.S.	N.S.	N.S.	0.02	N.S.	0.001

* Mean ±S.E., $n = 6$.
** Left anterior descending artery.
*** 1 kPa $\simeq$ 7.52 mmHg.
[a] Significant difference compared to control ($p < 0.05$).

vasoconstrictor action of the drug on stenosed coronaries. This is best envisaged by the last column of Table I. (To facilitate comparison, vascular conductance was expressed in percent, 100% being the value recorded before verapamil under normal conditions irrespective of the sequence of protocol.) The flow response to verapamil in the normal state was consistent with the well-known vasodilator potency of the drug. This vasodilation as contrasted with the decline of flow and vascular conductance in the stenosed coronaries after verapamil renders the difference between the two states highly significant. On the other hand, control values of all haemodynamic variables and blood pressure responses to verapamil were similar in both states.

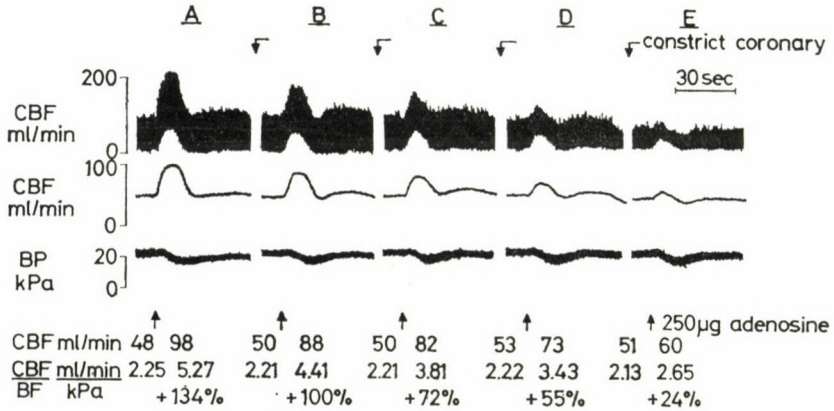

Fig. 1. Change of the adenosine effect (250 µg, left ventricle) during coronary constriction *A*, control; *B*—*E*, gradual mechanical constriction of the LAD artery. From top to bottom: phasic and mean records of coronary blood flow, blood pressure, numerical values for mean LAD flow and vascular conductance (flow/pressure), percent increases of vascular conductance

Experimental series II (cannulated vessels, 6 dogs)

Similar results were obtained when the whole left coronary hed was perfused through a bypass with the aid of an Eckstein cannula. Figure 2 depicts a typical experiment. As shown by the values for vascular conductance calculated using *post-stenotic* pressure (upper block), reversal of the vasodilator effect of verapamil in the constricted state was not due to the increase of the pressure drop through the mechanical stenosis. Statistical analysis of all experiments is shown in Table II. In this series a somewhat reduced propensity for autoregulation could be deduced from the moderate flow decline during mechanical constriction. This was probably caused by the more "unphysiologic" way of myocardial blood supply via the cannulated artery. Nontheless, only the flow changes *after* verapamil were consistent enough to reveal a significant difference between the normal and stenosed states.

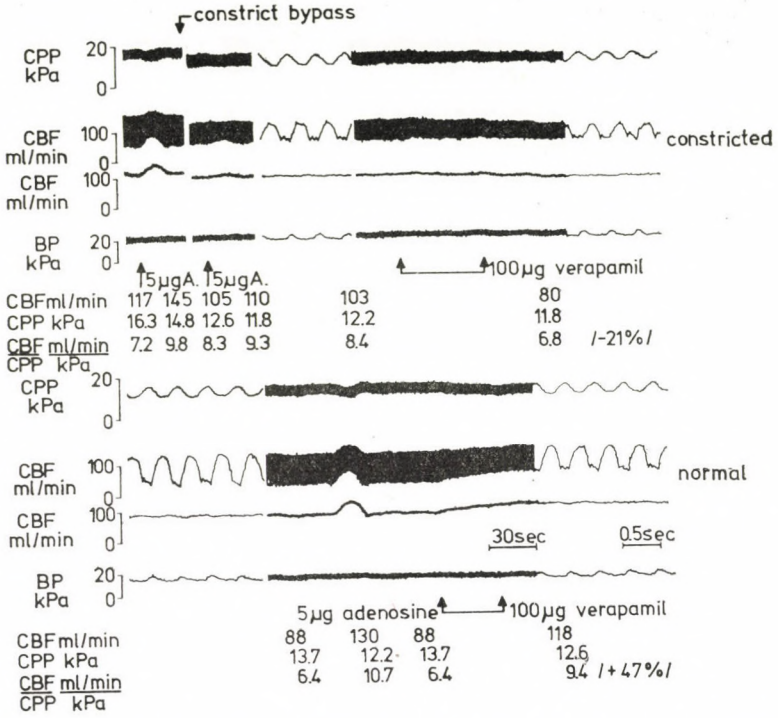

Fig. 2. Effects of adenosine (A) and verapamil in constricted (above) and normal (below) states of coronary circulation. Perfusion of the whole left coronary bed was effected from the carotid through an Eckstein cannula, CPP, coronary perfusion pressure (pressure head distal to the stenosis). Other symbols see in Fig. 1

Table II

*Effect of verapamil (8 µg/100 ml/min CBF, i.c.) on flow through coronary bypass**

	Arterial blood pressure (kPa)***		Coronary blood flow** (ml/min)		Vascular conductance (%)	
	Control	Verapamil	Control	Verapamil	Control	Verapamil
A Normal	17.4±1.1	16.4±1.0[a]	67.3± 6.9	97.8±17.5[a]	100 ± 0	151.6±14.5[a]
B Constricted	16.0±1.4	15.1±1.5[a]	56.9±10.1	44.9± 7.8	92.9±10.6	78.4± 9.2
P_{A-B}	N.S.	N.S.	N.S.	0.05	N.S.	0.01

* Mean ±S.E., $n=6$.
** Left common coronary artery.
*** 1 kPa ≃ 7.52 mmHg.
[a] Significant difference compared to control ($p < 0.05$).

Discussion

Verapamil, a vasodilator drug with a very marked coronary action, was found in this study to constrict rather than relax the resistance vessels of the critically stenosed coronary circulation. The possible explanation of this pharmacologic puzzle is best illustrated by the experiment shown in Fig. 3. Coronary vessels which were fully dilated under the influence of adenosine became *less* dilated after the subsequent administration of verapamil. When viewed from the control (pre-adenosine) state of the circulation, the coronaries are still relaxed after verapamil, a condition to be considered self-explanatory regarding the well-known vasoactive effects of both drugs. However, when viewed from the phase of the fully developed adenosine action, the coronary bed is constricted. It has been shown in previous experiments [4] that Ca-antagonists, although having a coronary dilator activity of their own, inhibit coronary vasodilation produced by both exogenous adenosine and metabolic coronary autoregulation, the latter phenomenon being probably associated with endogenous adenosine release from the heart muscle [1]. Any type of myocardial flow reduction enhances adenosine release thus promoting flow recovery, and this intrinsic feedback loop is notoriously resistant to pharmacologic interventions [5]. In contrast to methylxanthines (e.g. aminophylline) which block exogenous but not endogenous adenosine effects [6], the Ca-antagonists have the unique potency of inhibiting both phenomena simultaneously which render them useful investigative tools for suppressing metabolic coronary autoregulation as needed. Suppression of metabolic autoregulation is obviously potentiated by the verapamil-induced decline of the cardiac O_2

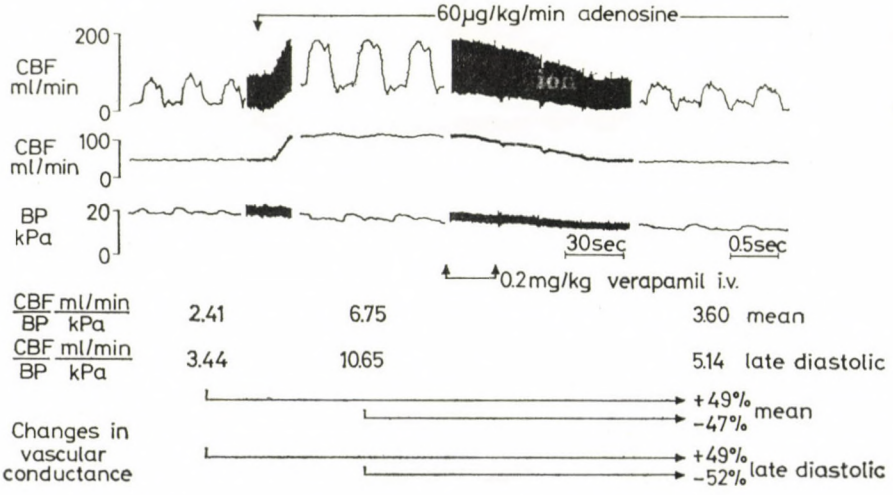

Fig. 3. Verapamil *decreases* vascular conductance on coronary vessels dilated with continuous adenosine infusion (left heart). Symbols as in Fig. 1. For explanation see text

demand and consequent adenosine release. These conclusions are summarized schematically in Fig. 4.

As far as we know, this strikingly differential action of Ca-antagonists on normal and stenosed coronaries has not been reported previously. However, an analogous reversal of coronary reactive hyperaemia has recently been described: Walinsky and his co-workers observed the actual decrease of flow following a momentary coronary occlusion with severe mechanical stenosis on the artery [7]. The authors hypothesized that this paradoxic reaction was caused by the hydrodynamic increase of the stenotic resistance, i.e. they postulated a BERNOULLI effect in the collapsible stenosed coronary branch. Although some increases of mechanical resistance cannot be excluded with certainly in our study either, three lines of evidence indicate that its contribution, if any, was not of decisive importance:

(a) In contrast with the reported study [7], drastic and abrupt changes of the intraluminal distending pressure, such as those occurring during reactive hyperaemia were carefully avoided.

(b) The verapamil effect in the stenosed coronaries, a response characteristically slow in onset, was never preceded by vasodilation which might have led to distal coronary pressure reduction.

(c) Finally, the pharmacologic effects obtained with the collapsible coronary artery (Series I), could be reproduced, with identical results, using a thick-walled, non-collapsible rubber tubing inserted into the coronary circuit (Series II).

We believe, therefore, that our results were due to the peculiar pharmacologic reactivity of the small coronary resistance vessels. If so, the described test might be useful to distinguish reactive coronary vessels from the coronary circulation compromised by atherosclerotic plaque formation.

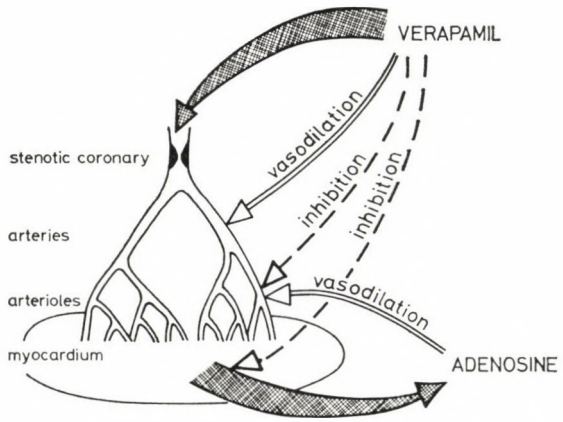

Fig. 4. Schematic representation of the proposed explanation for verapamil action on stenosed coronaries

The minimal hazard that might be involved in the adverse vascular actions of verapamil during this procedure, is amply counterbalanced by the well-known "protective" (antiarrhythmic and antiischaemic) effects of the drug exerted on the cardiac myocytes themselves. It seems worth emphasizing, however, that prior to the application of such a test in humans, its safety margins will need to be documented using very small doses.

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NICKEL INDUCES VASOCONSTRICTION IN THE ISOLATED CANINE CORONARY ARTERY BY A TONIC Ca^{2+}-ACTIVATION MECHANISM

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Nickel chloride ($NiCl_2$) at low concentration (1 μM) induced isometric force development in isolated canine coronary artery strips. The Ni^{2+}-action was linearly dependent on extracellular Ca^{2+}-concentration in the range of 0 to 5.0 mM. Verapamil (10^{-6}–10^{-3} M) did not prevent or abolish Ni^{2+}-induced coronary contraction, but nitroprusside sodium even at low concentration (10^{-8} M) antagonized the tonic force development. The results indicate that the stimulation of force development by trace amounts of $NiCl_2$ in isolated canine coronary artery strips is dependent on transmembrane Ca^{2+}-influx which is mediated by the T-system of Ca^{2+}-activation.

Nickel ions at low concentrations (10^{-8}–10^{-7} mol/l)-induced coronary vasoconstriction in the dog heart in situ and in the isolated perfused rat heart [6]. The Ni^{2+}-action in the rat heart was shown to depend on extracellular Ca^{2+}-concentration [6] and the coronary vasoconstriction induced by the trace metal could be abolished by the selective Ca-antagonist verapamil in both the dog and the rat heart [6]. Since changes in Ca^{2+}-concentration, and the use of Ca-antagonists were found to considerably influence myocardial function and metabolism [2, 5], these earlier experiments did not allow to differentiate between the direct action of Ni^{2+} on the coronary vessels and the secondary consequences of altered heart function and metabolism.

In order to study the direct vascular action of nickel ions and the Ca^{2+}-activation mechanism of Ni^{2+}-induced vasoconstriction, experiments were carried out on isolated canine coronary artery strips. The effect on Ni^{2+}-induced coronary vasoconstriction of changes in extracellular Ca^{2+}-concentration and the presence of various Ca^{2+}-antagonists was studied.

Materials and Methods

Mongrel dogs of either sex weighing 14 to 18 kg were anaesthetized with pentobarbital sodium (50 mg/kg; i.v.). The heart was rapidly excised and placed in ice-cold saline solution. The left anterior descending (LAD) coronary artery was carefully dissected from the free wall of the left ventricle and placed in mammalian Krebs–Ringer bicarbonate (KRB) solution of the following composition (mM/l): NaCl, 116; KCl, 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; dextrose, 10. In some experiments the Ca^{2+}-concentration of the solution was altered. The solution was bubbled by a 95% O_2 + 5% CO_2 gas mixture to give a pH of 7.4.

From the coronary artery segment (o.d. 1–1.5 mm) helical strips were cut in a counterclockwise fashion at an angle of 45°; they were approximately 1.5 mm wide and 15 mm long. The strip was suspended vertically in a water-jacketed thermostated (37 °C) glass chambe containing 20 ml of KRB. The distal end of the strip was anchored to the bottom of the chamber by a stainless steel hook and the proximal end was attached to a force transducer (GRASS FTO3C) to measure isometric force development. Isometric tension was recorded on a Radelkis recorder (Type OH-814/1). The strip was mounted between two platinum ring electrodes immersed 5 cm apart into the KRB solution. The electrodes served for electrical field stimulation; submaximal stimuli (10 to 15 V/5 cm; 50 Hz a.c., for 7 sec) were generated by a stimulator provided with an automatic timer device.

Results

As shown in Fig. 1, nickel chloride ($NiCl_2$) at 1 μM-concentration produced tonic contraction in the isolated canine coronary artery (Fig. 1A), which was not influenced by the presence of 10^{-3}M verapamil (Verpamil®, Orion) (Fig. 1B). Removal of external calcium (Ca^{2+}-free Krebs solution) abolished tension development in both strips. In contrast, phasic contractions evoked by electrical field stimulation were significantly depressed by verapamil (Fig. 1B).

The original recording in Fig. 2 illustrates the dependence on the extracellular Ca^{2+}-concentration of the electrically stimulated and 1 μM $NiCl_2$-induced isometric tension development. In Ca^{2+}-free Krebs solution neither field stimulation nor $NiCl_2$ caused an increase in isometric tension. The stepwise elevation of the external Ca^{2+}-concentration from 0.63 to 5.0 mM increased the amplitude of both the electrically stimulated phasic and the Ni-induced tonic contractions.

The tonic contraction evoked by $NiCl_2$ was not abolished or even depressed by verapamil, but the verapamil-resistant activation of the coronary artery could be antagonized by sodium nitroprusside at low concentration (10^{-8} M) (Fig. 3).

Discussion

The results showed that nickel ions produce tension development in the coronary artery by a direct action on vascular smooth muscle. Ni-activation was sensitive to calcium deprivation, and the Ni-induced stimulation of transmembrane calcium influx was resistant to verapamil but could be blocked by sodium nitroprusside.

A satisfactory interpretation of the results is possible on the basis of the "two calcium activation system" theory of GOLENHOFEN [3]. It is assumed that two chemically different systems for calcium activation exist in the membrane of the smooth muscle cell; they are called P- and T-systems. It is essential that both systems are dependent on transmembrane Ca^{2+}-fluxes [3].

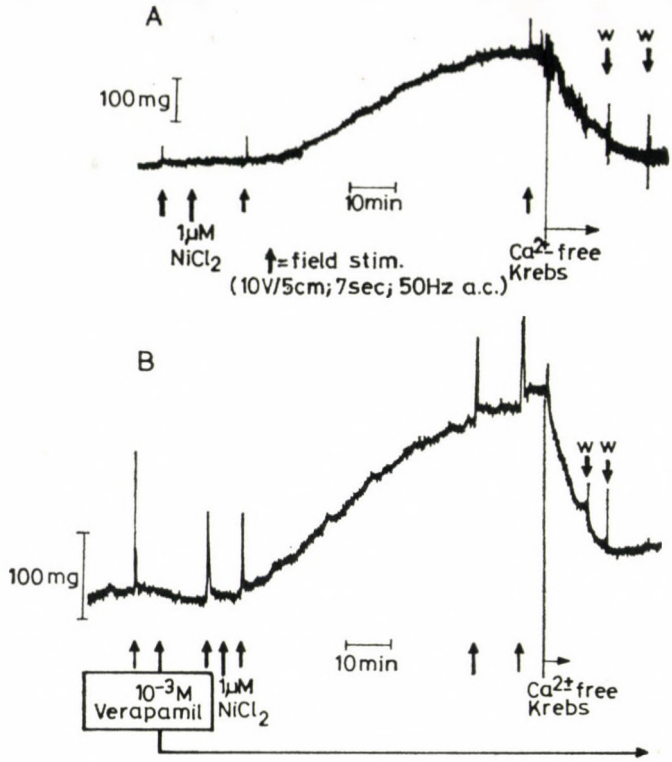

Fig. 1. A. Effect of $NiCl_2$ (1 μM) on basal tone and electrically stimulated isometric contractions in the isolated canine coronary artery. B. Effect of verapamil (10^{-3} M) on electrically stimulated phasic and Ni^{2+}-induced tonic isometric tension development in an isolated canine coronary artery strip. Note the rapid decline of tension in Ca^{2+}-free Krebs solution

The P-system is used for producing phasic activity, the T-system preferentially for tonic activity. The P-system can be blocked by verapamil, D-600 and nifedipine, while the T-system is resistant to these P-antagonists. In some smooth muscles, nitroprusside sodium proved to be a selective T-antagonist.

The present data suggest the existence of both the P- and T-systems of Ca^{2+}-activation in the membrane of the canine coronary vascular smooth muscle. Phasic contractions evoked by electrical stimulation were dependent on extracellular Ca^{2+}-concentration and they were significantly depressed by verapamil. In contrast, the tonic contraction induced by $NiCl_2$ was resistant to pretreatment or aftertreatment with verapamil, but was inhibited by the T-antagonist nitroprusside Na. A similar coexistence of the T-and P-systems was demonstrated in the guinea-pig uterus [3]. The present findings are consistent with the theory of GOLENHOFEN since the T-system activation by Ni^{2+} was also dependent on extracellular Ca^{2+}-concentration (Fig. 2). It was rapidly abolished in Ca^{2+}-free solution (Fig. 1) and Ni^{2+} failed to stimulate tension development in the absence of extracellular Ca^{2+} (Fig. 2). These results

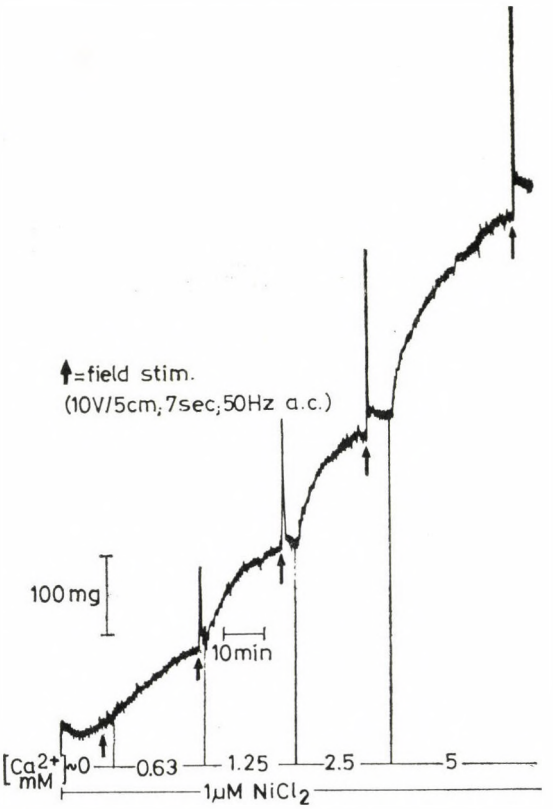

Fig. 2. Effect of stepwise elevation of extracellular Ca^{2+}-concentration on electrically-stimulated phasic and Ni^{2+}-induced tonic contraction in an isolated canine coronary artery

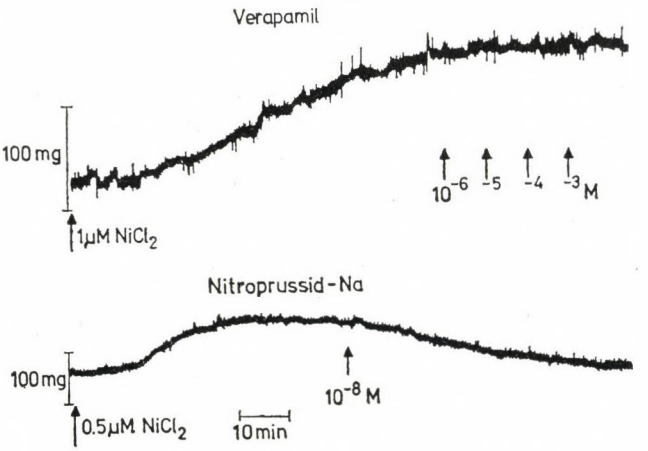

Fig. 3. Effect of verapamil and nitroprusside-Na on nickel-induced tonic tension development in an isolated canine coronary artery strip

indicate that activation of the T-system by nickel ions depends on the transmembrane Ca^{2+}-influx in the large canine coronary arteries. Ni^{2+} influences the P-system of the coronary artery as well, since the electrically stimulated phasic contractions were potentiated even before the tonic contraction had started to develop (Fig. 1A). This suggestion is substantiated by previous findings in that Ni^{2+}-induced coronary vasoconstriction was totally prevented by verapamil in the dog heart in situ, and in the isolated perfused rat heart [6]. Elevation of coronary resistance by Ni^{2+} in these preparations was assumed to be the consequence of Ni^{2+}-action on small arterioles rather than on large conductive coronary arteries. The activation of smooth muscle in the coronary resistance vessels (arterioles) by Ni^{2+} is most probably mediated by the P-system, which explaines the effectivity of verapamil and confirms the substantial functional differences between large and small coronary artery proposed by others [1, 4].

In conclusion, the action of Ni^{2+} on isolated large canine coronary artery is dependent on transmembrane Ca^{2+}-influx, and it is mediated by the T-system of Ca^{2+}-activation.

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THE ROLE OF ADRENERGIC RECEPTORS IN Ni^{2+}-INDUCED CORONARY VASOCONSTRICTION

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The possible involvement of adrenergic receptors in nickel ion (Ni^{2+})-induced coronary vasoconstriction was studied on isolated perfused rat hearts and on isolated canine coronary artery strips.

The experiments on both models showed that (i) alfa-adrenergic blockade by phenoxybenzamine or phentholamine caused only partial depression of Ni^{2+}-induced coronary vasoconstriction; (ii) beta-adrenergic receptor blockade by propranolol totally prevented Ni^{2+}-action, and (iii) Ni^{2+} (1 μM) caused significant inhibition of coronary vasodilatation induced by isoproterenol.

The experimental results indicate that alfa-adrenoceptors play minor role (if any) in the coronary action mechanism of Ni^{2+} but it may be mediated by beta-adrenergic mechanisms. Nickel was found to alter the reactivity of coronary beta-adrenoceptors suggesting a possible modulatory role of this trace metal in coronary adrenergic mechanisms.

The essential trace metal nickel ion (Ni^{2+}) was found to be a potent endogenous vasoactive agent [12], which induces coronary vasoconstriction in very low concentrations [9–14]. Its exact mechanism of action is unclear but there are data to suggest that adrenergic receptors in the coronary vessels may be involved in the action.

Nickel enhances Ca^{2+} influx into coronary vascular smooth muscle cells [13], similarly to alpha-adrenergic receptor stimulation [19].

A recent study revealed a close relationship between Ni^{2+}-induced coronary vasoconstriction and inhibition of the Na^+, K^+-pump [14]. In most vascular preparations, inhibition of the pump activity-induced vasoconstriction by stimulating norepinephrine release from intramural sympathetic postganglionic nerve endings [18]. The possibility thus exists that Ni^{2+}-action is connected with stimulation of neurotransmitter release and consequently with that of adrenergic receptors.

Nickel ions inhibit hypoxic/ischaemic coronary vasodilatation in the dog heart *in situ* [10, 11] and they abolish exogenous adenosine induced coronary vasodilatation in the isolated perfused rat heart [11]. Beside adenosine the stimulation of coronary beta-adrenergic receptors by catecholamines released from the hypoxic myocardium [15] may also contribute to the ischaemic/hypoxic changes of coronary resistance.

In order to analyse the nature of Ni^{2+}-induced coronary vasoconstriction and that of the inhibition of ischaemic coronary vasodilatation, the relationship between Ni^{2+} and coronary adrenergic mechanism has been studied.

Materials and Methods

Isolated perfused rat heart

White Wistar rats of either sex weighing 200 to 300 g were decapitated by a guillotine after an intraperitoneal injection of heparin (5 IU per g body weight). The hearts were rapidly removed and the aortic stump cannulated to allow retrograde coronary perfusion (modified Langendorff technique) by Krebs-Henseleit bicarbonate buffer solution (KHB) containing 10 mM glucose and 10 mU/ml insulin. The hearts were perfused by a constant flow peristaltic pump (Watson-Marlow) via babble-trap, thermostate (37 °C) and filter [9].

Mean perfusion pressure (PP) was measured just above the heart. All parameters were recorded on a Harvard Type 490 polygraph. Total coronary resistance (TCR) was calculated.

Isolated canine coronary artery strips

Mongrel dogs of either sex weighing 14 to 18 kg were anaesthetized with pentobarbital sodium (50 mg/kg; i.v.). The heart was rapidly excised and placed in ice-cold saline solution. The left anterior descending (LAD) coronary artery was carefully dissected from the free wall of the left ventricle and placed in mammalian Krebs-Ringer bicarbonate (KRB) solution of the following composition (mM/l): NaCl, 116; KCl 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; dextrose, 10.

The solution was aerated by a 95% O_2 + 5% CO_2 gas mixture to give a pH of 7.4.

Helical strips were cut from the coronary artery segment (o.d. 1–1.5 mm). The strips were cut in a counterclockwise fashion at an angle of 45°; they were approximately 1.5 mm wide and 15 mm long. The strip was suspended vertically in a water-jacketed, thermostated (37 °C) glass chamber containing 20 ml of KRB. The distal end of the strip was anchored to the bottom of the chamber by a stainless steel hook and the proximal end was attached to a force transducer (GRASS FTO3C) to measure isometric force development. Isometric tension was recorded by a Radelkis recorder (Type OH-814/1).

Chemicals

Norepinephrine (Noradrenalin; Gedeon Richter), isoproterenol (Isuprel^R, Winthrop); phenoxybenzamine (Smith, French and Kline); phentolamine (Regitine^R, CIBA), propranolol Inderal^R, ICI).

Statistical analysis

The means and standard error of the mean ($\bar{X} \pm$ SEM) of grouped experimental data were calculated. The statistical difference between means was estimated by paired and unpaired Student's t test.

Results

Isolated perfused rat heart

Increasing concentrations (0.01–100 μM/l) of $NiCl_2$ in the perfusate caused a significant rise of TCR (Fig. 1, control curve). The maximum effect was induced by 1 μM, and a half-maximum effect by 0.032 μM $NiCl_2$. Pretreat-

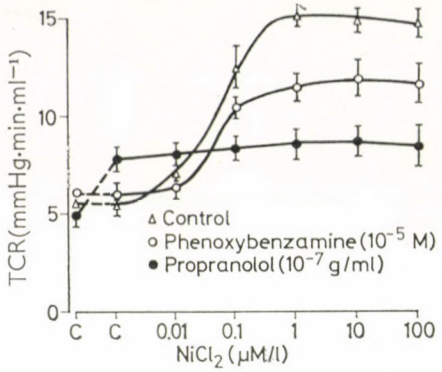

Fig. 1. Effect of phenoxybenzamine (10^{-5} M) ($n=6$) and propranolol (10^{-7} g/ml) ($n=6$) on basal coronary resistance (C') and on Ni^{2+}-induced coronary vasoconstriction (control = 10 hearts) in the isolated perfused rat heart ($\bar{x}\pm$S.E.)

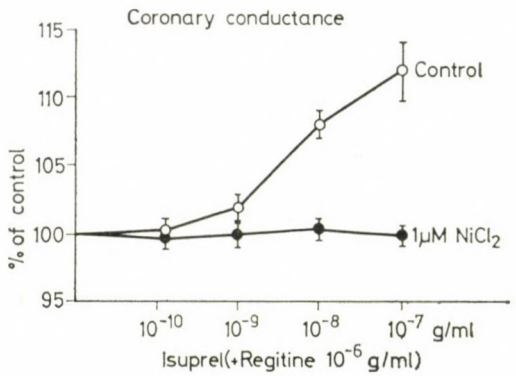

Fig. 2. Inhibition of isoproterenol induced coronary vasodilatation in the presence of phentol amine (10^{-6} g/ml) by 1 μM/l $NiCl_2$ in isolated perfused rat hearts ($n=5$) ($\bar{x}\pm$S.E.)

ment of the hearts by phenoxybenzamine (PBZ) (10^{-5} M) 30 min prior to Ni^{2+}-administration caused no change in basal coronary resistance but shifted the Ni^{2+}-response curve to the right and depressed the maximum action significantly ($p < 0.05$). The presence of 10^{-7} g/ml propranolol caused a significant rise of TCR and totally prevented Ni^{2+}-induced coronary vasoconstriction.

Stimulation of beta-adrenergic receptors in the presence of the alpha-receptor blocker phentolamine caused a dose-dependent increase in coronary conductance, which was totally abolished by 1 μM $NiCl_2$ in the perfusate (Fig. 2).

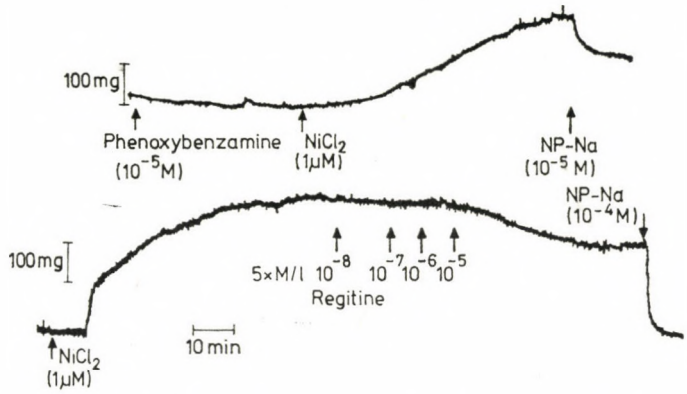

Fig. 3. Original record of the effect of alpha-adrenergic blockade on Ni^{2+}-induced isometric force development in isolated canine coronary artery strips. Top tracing: pretreatment by phenoxybenzamine (10^{-5} M). Lower tracing: effect of increasing doses of phentolamine on Ni^{2+}-induced coronary contraction. Force development was abolished by nitroprusside-Na (10^{-5} and 10^{-4} M, respectively) in both strips

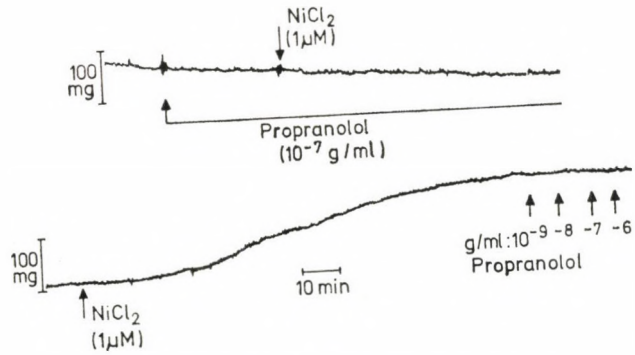

Fig. 4. Effect of pretreatment (upper tracing) and posttreatment (lower tracing) by propranolol on Ni^{2+}-induced isometric tension development in two isolated canine coronary artery strips. Note the total prevention of Ni^{2+}-action after pretreatment, but the ineffectiveness of post-treatment by the beta-receptor blocking agent

Isolated canine coronary artery

Pretreatment of the coronary artery strips by PBZ did not influence the development of Ni^{2+}-elicited isometric contraction (Fig. 3, upper tracing). Administration of phentolamine at the peak of isometric contraction evoked by 1 μM $NiCl_2$ caused only mild attenuation of the response and only in the highest concentration (10^{-5} M) of the alpha-receptor blocking drug (Fig. 3, lower tracing). Isometric force development could be abolished in both cases by nitroprusside sodium, the selective antagonist of tonic Ca^{2+}-activation mechanism [13].

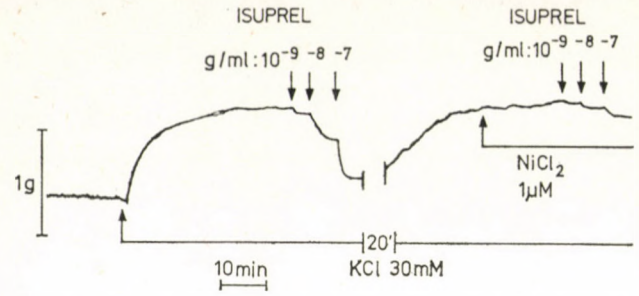

Fig. 5. Effect of increasing concentrations of isoproterenol on K^+-contraction (30 mM KCl) of an isolated canine coronary artery strip, in the absence and presence of 1 μM $NiCl_2$

Pretreatment of the isolated strips with the beta-adrenergic blocking agent propranolol (10^{-7} g/ml) totally prevented the development of Ni^{2+}-induced coronary vasoconstriction (Fig. 4, upper tracing). In contrast, administration of the blocking agent at the peak of isometric force development was ineffective, even in the highest concentration (10^{-6} g/ml) used (Fig. 4, lower tracing).

Beta-adrenergic receptor stimulation by increasing doses of isoproterenol of coronary arteries contracted by elevation of K^+-concentration in the medium (30 mM), caused a dose-dependent smooth muscle relaxation, which was almost totally abolished by 1 μM $NiCl_2$ (Fig. 5).

Discussion

The present experiments on isolated perfused rat hearts and isolated canine coronary arteries showed that (1) alpha-adrenergic blockade caused only partial inhibition of Ni^{2+}-induced coronary vasoconstriction; (2) beta-adrenergic receptor blockade totally inhibitied Ni^{2+}-action; and (3) Ni^{2+} caused a significant inhibition of coronary vasodilatation induced by beta-adrenergic receptor stimulation. These results suggest that adrenergic mechanisms are involved in the action of Ni^{2+} on coronary vessels. The stimulation of coronary alpha-adrenergic receptors was reported to induce coronary vasoconstriction in both the intact heart [6, 7] and the isolated coronary arteries [19]. Since Ni^{2+}-induced coronary vasoconstriction is also dependent on extracellular Ca^{2+}-concentration and transmembrane Ca-fluxes [9, 11, 12, 13], the possibility was raised that Ni^{2+} acts by stimulation of alpha-receptors by any of the following three mechanisms: (1) direct interaction with the receptor; (2) sensitization of the receptors to circulating catecholamines, and (3) stimulation of norepinephrine release from intramural nerve endings. The present findings that alpha-adrenergic receptor blockade causes only mild attenuation

of Ni^{2+}-induced coronary vasoconstriction indicates that alpha-receptor mechanisms play a minor role if any in the action mechanism of Ni^{2+}.

In contrast, Ni^{2+}-induced coronary vasoconstriction was totally inhibited by propranolol, and Ni^{2+} inhibited the effect of isoproterenol. These findings suggest that (1) Ni^{2+}-induced coronary vasoconstriction is mediated by beta-adrenergic mechanisms in the isolated rat heart and isolated canine coronary arteries, and (2) Ni^{2+} alters the reactivity of coronary beta-adrenoreceptors.

Under physiological conditions, catecholamines increase coronary blood flow [1, 20] and they induce coronary vasorelaxation by stimulating coronary beta-adrenoreceptors [2]. These previous observations have been confirmed by the present finding that propranolol caused a significant elevation of coronary resistance (Fig. 1), most probably by antagonizing the effect of catecholamines released from the heart [15].

There are, however, several reports in the literature which describe beta-adrenoreceptor mediated coronary vasoconstriction under certain pathological conditions. In conscious dogs with acute coronary occlusion, propranolol treatment has been shown to decrease coronary flow in the non-ischaemic myocardium and to augment perfusion in ischaemic zones [1, 20]. BORDA et al. demonstrated that a sudden elevation of K^+-concentration [3] and hypoxia [4] elicited sustained contractions in isolated canine coronary arteries which were inhibited by propranolol. SZENTIVÁNYI et al. [5, 16] described that norepinephrine evoked contractions in aorta strips isolated from diabetic rats, which could be antagonized by various beta-receptor blocking agents.

The mechanisms mediating these responses to beta-adrenergic stimulation have not been clarified. The hypoxic/ischaemic or diabetic alteration of beta-receptor reactivity may be explained on the basis of profound changes in vascular smooth muscle metabolism, which were shown to determine the interconversion of adrenoreceptors [8] by influencing the synthesis of modulator substances [17]. However, whatever the mechanism of altered beta-receptor activity, the present finding that Ni^{2+} alters beta-adrenergic reactivity in coronary vessels is of utmost importance, since significant endogenous Ni^{2+}-release was reported from the ischaemic-hypoxic myocardium [11, 12]. The possibility thus exists that endogenous Ni^{2+} may play some role in the ischaemic/hypoxic modulation of coronary beta-adrenoreceptors.

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DEPENDENCE OF NICKEL-INDUCED CORONARY VASOCONSTRICTION ON THE ACTIVITY OF THE ELECTROGENIC Na^+, K^+-PUMP

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The possible interactions between the vasoactive trace metal nickel ion (Ni^{2+}) and membrane Na—K-ATPase in the isolated perfused rat heart and in the isolated canine coronary artery have been studied. The characteristic features of 1 μM Ni^{2+}-induced contractile response in the canine coronary artery strip were similar to those evoked by the inhibition of Na—K-ATPase. Inhibition of the pump activity by ouabain (10^{-4} M) or by K^+-deficient Krebs solution prevented Ni^{2+}-action both in the canine coronary artery strip and in the perfused rat heart, indicating that when Ni^{2+} causes coronary vasoconstriction the Na, K-exchange is influenced. Further studies are needed to clarify whether Ni^{2+} acts directly on the enzyme, or the vascular action of this trace metal depends on the ionic gradients maintained by the electrogenic Na—K-pump.

Trace amounts (10^{-8}–10^{-7} M) of exogenous nickel chloride ($NiCl_2$) induce coronary vasoconstriction in the anaesthetized open-chest dog [16], in the isolated perfused rat heart [15] and in the isolated canine coronary artery [17]. Vascular action of Ni^{2+} is dependent on extracellular Ca^{2+}-concentration [15, 16] and Ni^{2+} enhances Ca-influx into vascular smooth muscle cells [15, 16, 17]. Among a number of possible mechanisms, the stimulation of Ca-influx by Ni^{2+} seems to be mediated by inhibition of membrane Na/K–ATPase, since previous studies showed that inhibition of the Na–K-pump by cardiac glycosides induced vasoconstriction [25] by stimulating Ca-influx [2, 25].

The present study was therefore designed to analyse the possible involvement of Na–K–ATPase inhibition in the action mechanism of Ni-induced coronary vasoconstriction in the isolated perfused rat heart and in the isolated canine coronary artery.

Materials and Methods

Isolated perfused rat heart

White Wistar rats of either sex weighing 200 to 300 g were decapitated by a guillotine after intraperitoneal injection of heparin (5 IU per g body weight). Hearts were rapidly removed and the aortic stump cannulated to allow retrograde coronary perfusion (modified Langendorff technique) by Krebs-Henseleit bicarbonate buffer solution (KHB) containing 10 mM glucose and 10 mU/ml insulin. The hearts were perfused by a constant flow peristaltic pump (Watson—Marlow) via bubble-trap, thermostat (37 °C) and filter [15].

Mean perfusion pressure (PP) was measured just above the heart. All parameters were recorded by a Harvard Type 490 polygraph. Total coronary resistance (TCR) was calculated.

Isolated canine coronary artery strips

Mongrel dogs of either sex weighing 14 to 18 kg were anaesthetized with pentobarbital sodium (50 mg/kg; i.v.). The heart was rapidly excised and placed in ice-cold saline solution. The left anterior descending (LAD) coronary artery was carefully dissected from the free wall of the left ventricle and placed in mammalian Krebs-Ringer bicarbonate (KRB) solution of the following composition (mM/l): NaCl, 116; KCl, 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; dextrose, 10.

The solution was aerated by a 95% O_2 + 5% CO_2 gas mixture to give a pH of 7.4.

Helical strips were cut from the coronary artery segment (o.d. 1–1.5 mm) in a counterclockwise fashion at an angle of 45°; they were approximately 1.5 mm wide and 15 mm long. The strip was suspended vertically in a water-jacketed thermostated (37 °C) glass chamber containing 20 ml of KRB. The distal end of the strip was anchored at the bottom of the chamber by a stainless steel hook and the proximal end was attached to a force transducer (Grass FTO3C) to measure isometrice force development. Isometric tension was recorded by a Radelkis recorder (Type OH-814/1).

The strip was mounted between two platinum ring electrodes immersed into the KRB solution 5 cm apart. These electrodes served for electrical field stimulation of the vascular strips. Submaximal stimuli (10 to 15 V/5 cm; 50 Hz a.c., for 7 sec) were generated by a stimulator provided with an automatic timer device.

Statistical analysis

The mean and standard error of the mean ($\bar{x} \pm$SEM) of grouped experimental data were calculated. The statistical difference between means was estimated by Student's paired and unpaired *t*-test.

Results

Characteristic features of Ni^{2+}*-action in isolated canine coronary artery*

The typical original recording in Fig. 1 demonstrates some of the characteristic features of the contractile response induced by 1 μM $NiCl_2$ in the canine coronary artery strip. These are, potentiation of electrically-stimulated isometric force development; induction of slowly rising tension development without stimulation after a long latency period; and the interruption of force development by Ca^{2+}-free solution.

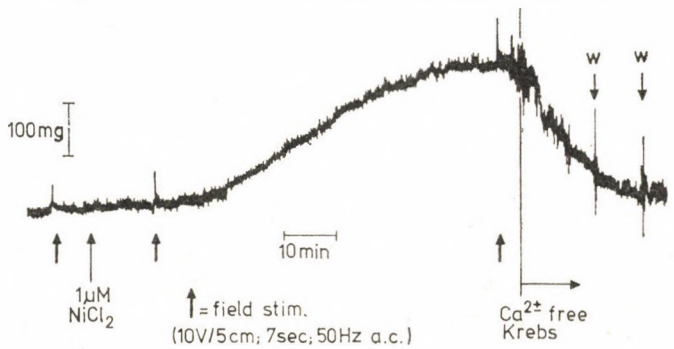

Fig. 1. Effect of $NiCl_2$ (1 μM/l) on isolated canine coronary artery. The typical original recording demonstrates that Ni^{2+} potentiates electrically-stimulated phasic contractions and evokes tonic contracture without stimulation, which is abolished by Ca^{2+}-free Krebs solution

Effect of Na–K–ATPase *inhibitors on* Ni^{2+}*-induced coronary vasoconstriction*

Increasing concentrations (0.01–100 μM/l) of $NiCl_2$ in the perfusate induced a significant TCR elevation (Fig. 2) in the isolated rat heart. Maximum effect was reached after the addition of 1 μM $NiCl_2$ while a half-maximum TCR increase was achieved by a Ni^{2+} concentration as low as 0.032 μM/l. Addition of 10^{-4} M ouabain (strophanthin G; Calbiochem) induced an almost 100% increase in coronary resistance and the subsequent administration of $NiCl_2$ was ineffective. Replacement of the normal perfusate with K^+-deficient KRB (K^+: 1.2 mM), also increased coronary resistance by approximately 35%. Nickel caused a further increase of TCR in K^+-deficient KRB, but its maximum effect was significantly ($p < 0.05$) depressed.

Ouabain (10^{-4} M) caused isometric force development in the isolated canine coronary artery strip after a long latency period (Fig. 3). Addition of 1 μM $NiCl_2$ to the muscle bath at the peak of the ouabain-contraction did not evoke any further force development. Repeated administration of $NiCl_2$ after 60 min incubation of the strip in ouabain-free solution induced contraction similar to that observed under control conditions (see Fig. 1).

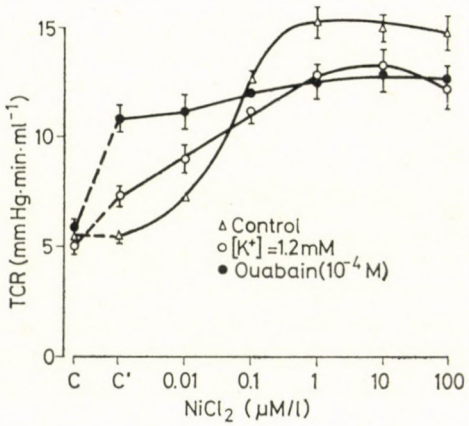

Fig. 2. Effect of ouabain (10^{-4} M) and K^+-deficient (1.2 mM) Krebs solution on Ni^{2+}-induced elevation of total coronary resistance (TCR) in the isolated perfused rat heart ($\bar{x} \pm$S.E.)

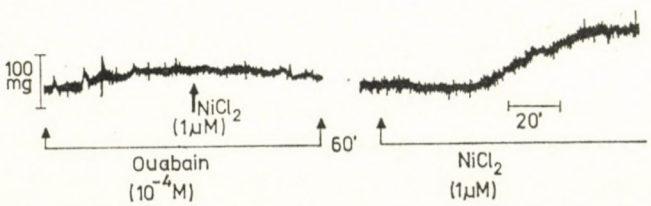

Fig. 3. Effect of ouabain (10^{-4} M) on basal tone and Ni^{2+}-action in isolated canine coronary artery

Discussion

The active transport of Na^+ and K^+ across the cell membrane is linked to the enzyme Na–K–ATPase, which is inhibited by cardiac glycosides and K^+-free and Na^+-free solutions [19]. Activation of Na–K–ATPase in isolated blood vessels causes relaxation which has been atributed to hyperpolarization of the cell membrane [5, 6, 9, 22, 26], while inhibition of the pump causes vasoconstriction most probably as a consequence of membrane depolarization [1, 20, 21] and increase of Ca-influx [2]. The present study confirmed the vasoconstrictive property of ouabain and K^+-deficient solution in the canine and rat coronary artery.

Experimental evidences suggest that the Na–K–ATPase plays important role in the action mechanism of Ni^{2+}-induced coronary vasoconstriction. The first (indirect) evidence is based on the finding that the characteristic features of Na–K-pump inhibition and Ni^{2+}-induced coronary-vasoconstriction are very similar.

Cardiac glycosides were found to potentiate electrically-stimulated vascular contractions [7, 13] and after some delay induced tension without stimulation in various isolated blood vessels [3, 4, 7, 8, 24]. The ouabain-induced contraction of the rabbit aorta [8, 24] and canine coronary artery is accompanied by an increase in Ca^{2+}-uptake, and both the contraction and the stimulated Ca-influx were abolished by selective Ca-antagonists or by removal of external Ca^{2+}. These properties of ouabain show a striking similarity to those of Ni^{2+}-induced coronary vasoconstriction (see Fig. 1).

The possible involvement of Na–K-pump inhibition in the action mechanism of Ni^{2+} is further substantiated by the fact that ouabain totally prevented Ni^{2+}-induced coronary vasoconstriction both in the isolated perfused rat heart (Fig. 2) and in the isolated canine coronary artery (Fig. 3). These results are consistent with the interpretation that when Ni^{2+} causes constriction of coronary vascular smooth muscle cells, the Na^+, K^+ exchanges are involved. Further studies are needed to clarify whether Ni^{2+} acts directly on the enzyme and/or the vascular action of this trace metal depends on the ionic gradients maintained by the electrogenic Na–K-pump [1].

Several observations indicate that most of the contractile effects elicited by inhibition of Na–K–ATPase are neurogenic in origin, since these contractions are abolished by alpha-adrenergic blockade and chemical denervation [6, 12, 24]. In addition, BONACCORSI et al. [6] showed a close correlation between the time course of delayed contraction and zero K^+-induced release of ^3H-labelled norepinephrine from the nerve endings of the rat tail artery. The possibility thus exists that Ni^{2+}-induced and Na–K-pump dependent coronary vasoconstriction is mediated by neurotransmitter (catecholamine) release and therefore by stimulation of the vascular adrenergic receptors. This problem was analysed in a recent study [10].

In the saphenous vein and basilar artery of the dog [11] and in the rat portal vein [18], unlike in the canine femoral [11] and coronary artery [15–17], nickel ions have no stimulatory action but they rather reduce the basal tone and depress electrically-stimulated contractions [11, 18]. The present study suggests that the vasoconstrictor effect of Ni^{2+} depends on the activity of the electrogenic Na–K-pump and variations in the functional importance of this membrane process [23] could provide an explanation for the heterogeneous responses to Ni^{2+} of blood vessels of different origin.

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EFFECT OF Ni^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+} AND VO_3^- ON CORONARY VASCULAR RESISTANCE IN THE ISOLATED PERFUSED RAT HEART

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The coronary vascular effect of various divalent cations and of sodium-meta-vanadate was compared in isolated perfused rat hearts. Their order of activity was as follows (the concentrations (μM) evoking a half-maximum increase of coronary resistance are indicated (in parentheses): Ni^{2+} (0.03) > Co^{2+} (0.1) > Hg^{2+} (0.16) > VO_3^- (0.2) > Cu^{2+} (15) > Zn (50). Iron (Fe^{2+}) and cadmium (Cd^{2+}) were ineffective. The order of coronary vasoconstrictor potency of these metal ions differs from the order of their other physico-chemical properties indicating that their coronary action cannot be explained as being singly a consequence of ion-membrane interaction. In contrast to Ni^{2+}, the effect of Hg^{2+} was totally inhibited by phenoxybenzamine (10^{-5} M) indicating that coronary vasoconstriction induced by mercury ions is mediated by alpha-receptors. Coronary vasoconstriction induced by sodium-meta-vanadate was resistant to verapamil while removal of external Ca^{2+} potentiated its effect.

These data suggest that in contrast to Ni^{2+} and Hg^{2+}, vanadate increases coronary resistance by mobilizing intracellular Ca^{2+} in vascular smooth muscle cells.

RUBÁNYI et al. [5] showed that $NiCl_2$ in very low (micromolar) concentration constricts coronary arteries of the isolated perfused rat heart. Vanadate (VO_3^-) [8] and mercury [2] ions in micromolar concentrations also elevate the coronary resistance in the isolated rat heart. Prolonged administration of $CdCl_2$ causes hypertension in rats and rabbits and cardiovascular lesions were observed in humans after exposition to Cd [7]. The effect of other trace elements on coronary circulation is not known.

Ni ions easily form complexes with various ligands occurring on the surface of biological membranes [2]. We assume that its biological effect is due to the ion-membrane interaction of unknown origin. To clarify this hypothesis we compared the effect of Ni^{2+} on coronary circulation with that of the other trace elements belonging to the transition group: cobalt (Co), copper (Cu), zinc (Zn), cadmium (Cd), iron (Fe), vanadium (as vanadate anion VO_3^-) and mercury (Hg). Furthermore, the coronary vascular action of Ni^{2+} was compared to that of the well-known SH-reagent Hg^{2+} [1] and of the Na, K–ATPase inhibitor vanadate [4] in the presence of the selective Ca-antagonist verapamil and the alpha-blocker compound phenoxybenzamine (PBZ), and also in the absence of extracellular Ca^{2+}.

Methods

White laboratory rats of either sex weighing 200 to 300 g were decapitated after i.p. injection of heparin (5 IU/g b.w.). After rapid removal of the heart the coronary system was perfused through the cannulated aorta (modified Langendorff technique) with Krebs–Henseleit bicarbonate solution (pH=7.3–7.4) containing 10 mM glucose and 10 mU/ml insulin [5].

The heart was perfused by a constant flow peristaltic pump (Harvard, Type 1210) via a bubble-trap and a filter at room temperature. Mean perfusion pressure was measured by a pressure-transducer (Sanborn, Type 267BC) and coronary flow was determined by a syringe under the heart. Pressure was recorded continuously (Harvard polygraph, Type 490). Total coronary resistance was calculated. The TCR-elevating effect of the ions studied was compared according to their concentration in the perfusate (μmol/l) which elicited half-maximum vasoconstriction. This value was called ED_{50}.

The chemicals used in the experiments were verapamil (Verpamil®, Orion) and phenoxybenzamine (Smith Kline and French). Values given are the means ±SEM. Data were analysed for statistical significance by Student's *t*-test.

Results

The action of the studied elements on coronary resistance in the isolated rat heart is summarized in Fig. 1. The following ions caused an elevation of TCR: Ni^{2+}, Co^{2+}, Hg^{2+}, VO_3^-, Cu^{2+} and Zn^{2+}, while Fe^{2+} and Cd^{2+} did not induce any change. The sequence of activity of the elements calculated on the basis of ED_{50} was, Ni^{2+} (0.03 μmol/L) > Co^{2+} (0.1) > Hg^{2+} (0.16 > VO_3^- 0.2) > Cu^{2+} (15) > Zn^{2+} (50). The Ni^{2+}-induced rise of TCR was completely

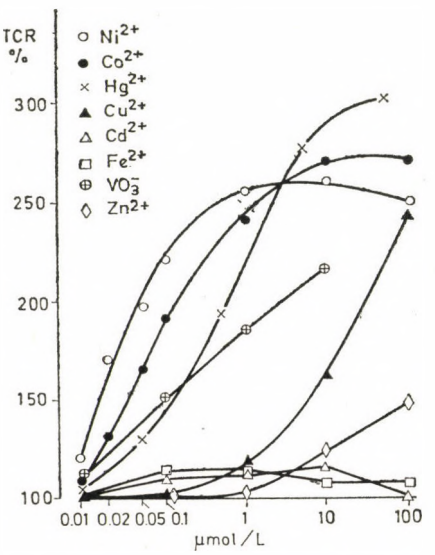

Fig. 1. Effect of increasing doses (0.01–100 μmol/l) of $NiCl_2$ (n=8), $CoCl_2$ (n=5), $HgCl_2$ (n=8), $CuCl_2$ (n=4), $NaVO_3$ (n=4), $ZnSO_4$ (n=5), $CdCl_2$ (n=4), $FeCl_2$ (n=4) on total coronary resistance (TCR) expressed in per cents of the control in the isolated perfused rat heart

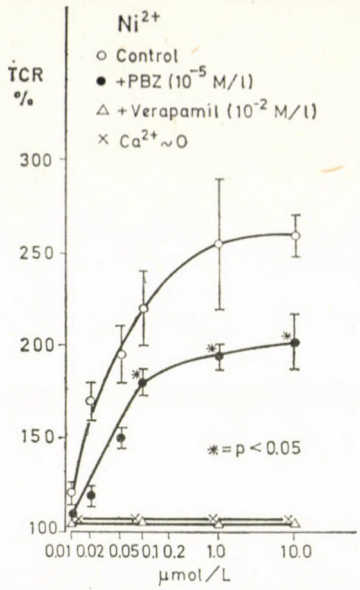

Fig. 2. Effect of verapamil (n=4), PBZ (n=5) and Ca-deficient medium (n=4) on Ni^{2+}-induced TCR elevation in the isolated rat heart

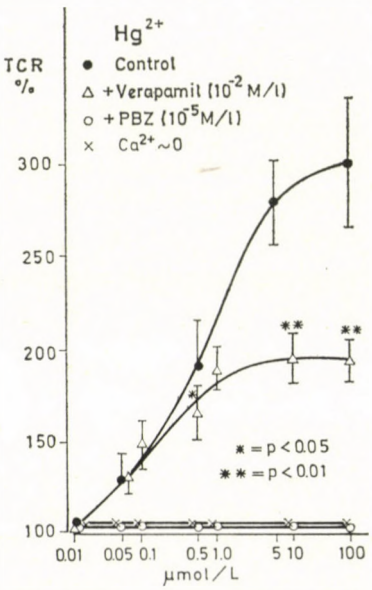

Fig. 3. Effect of verapamil (n=5), PBZ (n=5) and Ca-deficient medium (n=5) on Hg^{2+}-induced TCR elevation in the isolated perfused rat heart

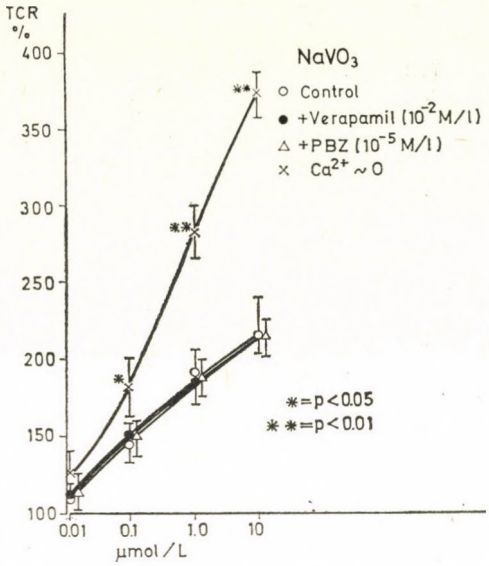

Fig. 4. Effect of verapamil (n=5). PBZ (n=5) and Ca-deficient medium (n=4) on VO_3^--induced TCR elevation in the isolated rat heart

inhibited in the Ca-deficient medium as well as after treatment with verapamil (10^{-2} M/l). PBZ (10^{-5} M/l) shifted the dose-response curve to the right and depressed the maximum effect significantly, indicating a partial inhibition of the Ni^{2+}-action (Fig. 2). $HgCl_2$ also failed to cause coronary vasoconstriction in Ca-deficient medium but verapamil treatment only partially inhibited the coronary effect (Fig. 3). After treatment with PBZ, coronary vasoconstriction was completely abolished.

Vanadate ion caused a linear rise of TCR in the 0.01–10 µmol/l concentration range (Fig. 4). Neither verapamil, nor PBZ did alter this response, while in Ca-deficient buffer there was a significant enhancement of the TCR-increasing action of VO_3^-.

Discussion

Comparative study showed that like Ni^{2+}, the divalent cations Co^{2+}, Hg^{2+}, VO_3^-, Cu^{2+}, Zn^{2+} also elevate TCR in the isolated perfused rat heart. Iron and cadmium ions in the applied concentration range (0.01–100 µmol/l) did not change TCR. The obtained values of ED_{50} indicate the following order of activity: $Ni^{2+} > Co^{2+} > Hg^{2+} > VO_3^- > Cu^{2+} > Zn^{2+}$, which differs markedly from any other order of chemical activity (see Table I).

Bers and Langer [3] found that in the cardiac sarcolemma the Ca-displacing activity of divalent uncoupling ions increases with the ionic radius.

Table I

Comparison of coronary vasoconstrictor potency with the order of several chemical properties of the metal ions studied

Coronary vasoconstrictor activity	$Ni > Co > Hg > VO_3 > Cu > Zn$
Atomic number	$Co > Ni > Cu > Zn > Cd > Hg$
Solubility constant (—SH)*	$Hg > Cu > Cd > Ni > Zn$
Stability constant (ligand $-NH_3$)*	$Hg > Cu > Zn > Ni > Cd > Co$
Ionic radius*	$Hg > Cd > Zn > Co = Cu > Ni$

* According to Århem [1].

In contrast, the coronary vasoconstrictor potency of the studied metal ions differed fundamentally from the sequence of the ionic radii and other chemical properties listed in Table I. These experiments thus suggest that the coronary vasoconstrictor effect is not a simple consequence of ion-membrane interaction.

The effect of nickel is totally abolished in Ca-deficient medium and by verapamil, confirming earlier results [6] showing that Ni^{2+} increases Ca-influx into the coronary vascular smooth muscle cells. One of the possible mechanisms of such an enhancement of Ca-transport may be the activation of alpha-receptors in the coronary vessels [9]. PBZ-pretreatment of the isolated heart inhibited the vasoconstrictive effect of Ni^{2+} only partially, indicating that other mechanisms are also involved.

Similarly to that of nickel, the coronary vasoconstrictor action of the mercury ion closely depends on the presence of extracellular Ca^{2+}. At the same time, verapamil inhibited only partially the action of Hg^{2+}. The finding suggests that the action of Hg^{2+}-action on the coronary vessels depends only partly on the activation of verapamil-sensitive Ca-channels. The finding that PBZ totally abolished the action of Hg^{2+} indicates that Hg^{2+}-induced coronary vasoconstriction in the rat heart is mediated by alpha adrenergic receptors.

The characteristic features of vanadate-induced coronary vasoconstriction differ basically from those elicited by Ni^{2+} and Hg^{2+}. Verapamil and PBZ-treatment did not influence TCR changes induced by VO_3^-. In addition, removal of extracellular Ca^{2+} significantly potentiated its effect. It is assumed that VO_3^- activates coronary vascular smooth muscle contraction by a mobilization of intracellular Ca^{2+}.

The difference of the coronary action of these three ions indicates that the Ni^{2+}-effect cannot be explained only by an interaction with SH-groups or with Na, K–ATPase in the isolated rat heart.

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EFFECT OF NICKEL IONS ON SPONTANEOUS, ELECTRICALLY AND NOREPINEPHRINE STIMULATED ISOMETRIC CONTRACTIONS IN THE ISOLATED PORTAL VEIN OF THE RAT

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The effect of low concentrations (1–10 μM) of nickel (Ni^{2+}) was studied on the spontaneous contractile activity of isolated rat portal vein preparations. To distinguish between presynaptic and postsynaptic actions, Ni^{2+}-induced changes of isometric force development evoked either by electrical field stimulation (80V/5 cm; 1 msec; 1–32 Hz) or by exogenous norepinephrine (NE) were compared.

Ni^{2+} inhibited spontaneous isometric force development, decreased basal tone but significantly increased the frequency of contractions. The experimental findings that inhibition of the effect of selective stimulation of adrenergic nerves was significantly more pronounced than the depression of contractions evoked by exogenous norepinephrine indicate that in addition to postsynaptic actions, Ni^{2+} influences presynaptic mechanisms (i.e. NE-release) in this vessel preparation.

Nickel ions (Ni^{2+}) are potent endogenous vasoactive agents [10] which induce coronary vasoconstriction in the isolated rat heart [8], in the in situ dog heart [9] and in the isolated canine coronary artery [11]. The last mentioned preparation is most suitable for analysis of the direct action of Ni^{2+} on vascular smooth muscle cells with certain limitations. The most important factor in this respect is the fact that the smooth muscle of large "Windkessel" and conduit arteries (which are primarily suited for this type of study for technical reasons) differ in several functional characteristics from the smooth musculature of the haemodynamically more important small peripheral resistance vessels [3]. In fact, previous studies have revealed several differences between Ni^{2+}-induced elevation of coronary resistance in the intact heart and coronary vasoconstriction in the isolated large coronary artery strips [11].

The portal vein proved to be a valuable model for studying the characteristics of peripheral circulation where the vessels are less accessible to direct investigations [7]. The present study was therefore designed to investigate the effect of low concentrations (1 and 10 μM/l) of nickel ions on various parameters of the spontaneous contractile activity of the isolated rat portal vein. To distinguish between presynaptic neurotransmitter release and postsynaptic smooth muscle actions of this trace metal Ni^{2+}-induced changes of isometric force development evoked either by selective electrical field stimulation or by exogenous norepinephrine (postsynaptic) stimulation were compared.

Methods

Experiments were performed on 10 portal vein preparations from rats of the Wistar strain weighing 200–300 g. The animals were decapitated by guillotine, exsanguinated, the portal veins dissected, removed within 2 min after decapitation and placed into oxygenated Krebs–Ringer bicarbonate solution (KRB) of the following composition (mM/l): NaCl: 116; KCl: 4.6; $CaCl_2$: 2.5; KH_2PO_4: 1.2; $MgSO_4$: 1.2; $NaHCO_3$: 25 and glucose 10. The solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2 to give a pH of 7.4.

The 15–20 mm long vascular segments were suspended vertically in a water-jacketed thermostated (37±0.2 °C) plexy chamber containing 20 ml of KRB. The mechanical activity of the longitudinal musculature of the portal vein was recorded isometrically with a force — displacement transducer (Grass FTO3C) operating an inkwriting recorder (Esterline Angus). The passive tension applied to the muscle varied between 800 and 1000 mg. The muscle at this passive tension was allowed to recover for about 1 h in the bath.

Nerve responses were obtained by selective activation of the intramural adrenergic nerve plexus by means of transmural electrical field stimulation. A Grass S-48 stimulator provided square wave impulses of supramaximal amplitude (80 V) and 1 msec duration to platinum ring electrode mounted in the wall of the muscle chamber 5 cm apart.

Experimental protocol

Frequency-response relationships were obtained by field stimuli of 2, 4, 8, 16 and 32 HZ frequency lasting for 1 min at 3 min intervals. After stimulation, when the baseline had completely returned to the resting level, exogenous norepinephrine (NE) was injected into the bath to give a final concentration of 10^{-7} g/ml. Afterwards NE was washed out and 15 min later $NiCl_2$ was injected into the bath to give a final concentration of 1 μM/l. After the Ni^{2+} effect had became stable (~30 min), the electrical and NE stimulations were repeated. The effect of 10 μM/l $NiCl_2$ was similarly studied on the same preparation.

The mean and standard error of the mean ($\bar{x}$±S.E) of grouped experimental data were calculated. Comparison of mean values was performed by Student's *t*-test.

Results

The typical original recordings in Fig. 1 demonstrate the rhythmic spontaneous contractile activity and the frequency-response relationship induced by transmural field stimulation in an isolated rat portal vein preparation under control conditions and in the presence of 1 and 10 μM/l $NiCl_2$. Basal tone (i.e. tension measured in the relaxed state of the muscle) and the amplitude of spontaneous phasic contractions were significantly reduced by 1 μM $NiCl_2$ (Fig. 2) but there was a significant increase observed in the frequency of spontaneous contractions (Fig. 2). Elevation of Ni^{2+}-concentration from 1 to 10 μM/l caused no significant changes in any of these parameters. The mean values of transmural field-stimulation-induced frequency-response relationships are illustrated in Fig. 3. Electrical field-stimulation caused a steep rise of tension in the low frequency range (2–8 Hz) which was followed by a much slower increase at higher frequencies (16 and 32 Hz) under control conditions. $NiCl_2$ in 1 μM concentration caused a significant depression of the curve at every frequency value studied, and the presence of 10 μM $NiCl_2$ in the bath caused a further significant inhibition. Exogenous norepinephrine

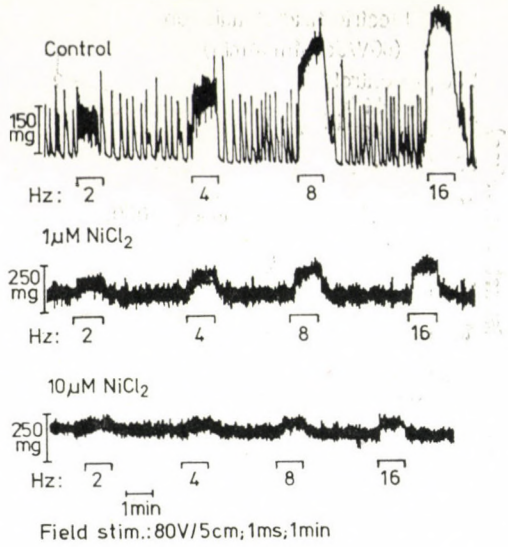

Fig. 1. Spontaneous and electrically-stimulated isometric contractions of an isolated rat portal vein preparation. Note the significant inhibition of mechanical activity by 1 and 10 μM/l $NiCl_2$

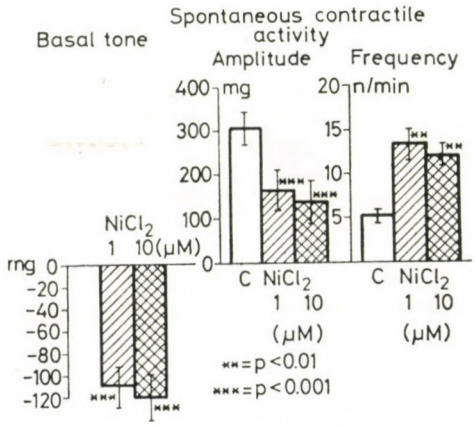

Fig. 2. Effect of 1 and 10 μM/l $NiCl_2$ on basal tone and amplitude and frequency of spontaneous contractions of rat portal veins ($n=10$) ($\bar{x}\pm$S.E.)

in a concentration of 10^{-7} g/ml, caused isometric force development (Fig. 4), the magnitude of which was approximately equivalent to that seen after 32 Hz electrical stimulation (Fig. 3). Addition of 1 μM $NiCl_2$ to the muscle bath depressed the NE response as well, but the extent of inhibition (15–20%) was significantly less pronounced than that observed in the case of 16 and 32 Hz electrical stimulation (60–65%).

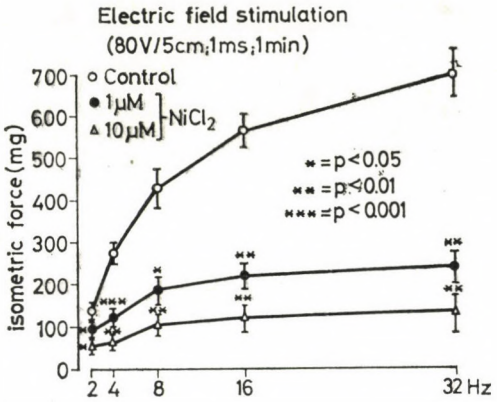

Fig. 3. Effect of 1 and 10 μM/l $NiCl_2$ on the frequency-response relationship evoked by electrical field stimulation in rat portal vein (n=10 ($\bar{x} \pm$S.E.)

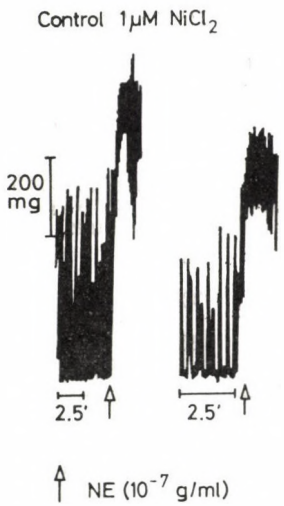

Fig. 4. Isometric contractions induced by exogenous norepinephrine (10^{-7} g/ml) in rat portal vein preparation in the absence (control) and presence of 1 μM/l $NiCl_2$

Discussion

The present study on the spontaneously active isolated rat portal vein showed that nickel ions in low concentrations (1 and 10 μM/l) inhibited both the spontaneous and electrically — as well as exogenous norepinephrine — stimulated isometric contractions. These actions are opposite to the vasoconstriction induced by Ni^{2+} in the isolated canine coronary [11] and femoral arteries [5] but they are similar to the Ni^{2+}-induced inhibition of contractile

activity in the isolated canine basilar artery and saphenous vein [5]. The exact nature of these basically different actions of the same trace metal in various blood vessels is unexplained but confirms earlier findings [2] in that functional characteristics of vascular smooth muscle in blood vessels of different anatomical origin are heterogenous. Thus, the future analysis of the action mechanism of Ni^{2+} may help to elucidate the functional heterogeneity of vascular smooth muscle as well.

KOHLHARDT et al. [6] have recently suggested, on the basis of experiments with tension recording and Ca^{2+}-flux data in isolated guinea-pig ventricular strip preparations, that the Ni^{2+}-induced inhibition of cardiac contractility operates by an antagonism between nickel and calcium ions on superficial binding sites. This is of interest with regard to the present observations on the portal vein where the inhibitory response to nickel ions resembles in its mechanical components the activity changes produced by lowering the Ca^{2+}-concentration in the medium [1]: reduction of the Ca^{2+}-concentration to 0.5–0.6 mM caused a decrease of both the basal tone and the amplitude of spontaneous contractions with a parallel significant increase of the frequency of contractions (see also Figs 1 and 2). These data suggest that Ni^{2+} acts as a Ca^{2+}-antagonist in the isolated rat portal vein.

The present finding that the Ni^{2+}-induced inhibition of contractions evoked by selective stimulation of intramural adrenergic nerves was significantly more pronounced (Figs 1 and 3) than the depression of contractions stimulated by exogenous norepinephrine (Fig. 4) indicate that in addition to its postsynaptic actions (i.e. on the vascular smooth muscle cell membrane) Ni^{2+} influences a presynaptic mechanism, i.e. NE-release from the sympathetic nerve endings. It is proposed that Ni^{2+} inhibits NE-release from the nerves by antagonizing Ca^{2+}-influx, but other mechanism may also be involved. More detailed studies are required to clarify the possible role of nickel ions in adrenergic neurotransmission. The finding that the response to NE was more insensitive to Ni^{2+} than were spontaneous contractions, may be explained by the fact that NE induces contraction in the portal vein by mobilizing intracellular Ca^{2+} [4], while spontaneous contractions depend primarily on the influx of extracellular Ca^{2+} into smooth muscle cells [1].

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INHIBITION OF MYOCARDIAL REACTIVE HYPERAEMIA BUT NOT OF HYPOXIC CORONARY VASODILATATION BY CONSTANT VERAPAMIL INFUSION IN THE IN SITU DOG HEART

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The purpose of the present study was to investigate the changes of characteristic parameters of myocardial reactive hyperaemia that follow coronary artery occlusion for 10 sec and of the coronary vascular response to hypoxia during constant verapamil infusion (0.01 mg. kg^{-1}. min^{-1}) in anaesthetized open-chest dogs. Reactive hyperaemia was significantly reduced by the specific Ca-antagonist verapamil, while augmentation of coronary blood flow by 100% N_2 inhalation for 30 sec was not significantly influenced by the drug. The experimental results indicate that, similar to myocardial cells, verapamil reduces the oxygen requirement of vascular smooth muscle cells as well. The possibility exist that verapamil excludes factor(s) responsible for the disproportional overpayment of flow debt.

Numerous studies on coronary circulation have indicated that local factors within the heart are of primary importance in changing the basal tone and thereby adjusting coronary blood flow (CBF) to meet the oxygen requirement of the myocardium [2]. Reactive hyperaemia (RH) of the coronary vessels following mechanical occlusion of various duration has been proved to be a valuable experimental model for the study of local regulation of CBF [10]. Previous studies have revealed that RH in the heart is a complex response having both physical and chemical determinants (e.g. locally produced vasodilatory metabolites, vascular smooth muscle oxygen deprivation and loss of myogenic tone during coronary occlusion) [10]. Adenosine was proposed as a key factor in the metabolic regulation of CBF [3] and has been demonstrated to be released from the normally oxygenated heart [9, 11].

The demonstration that adenosine is released from the heart in response to increased myocardial oxygen demand [3, 8] support the assumption that adenosine plays a key role in the coupling of myocardial metabolism to CBF. Numerous studies have shown that organic Ca-antagonists (e.g. verapamil) decrease myocardial oxygen demand [5], thus, investigation of the effects of verapamil on the RH of coronary vessels may provide additional evidence for the role of metabolism-dependent adenosine accumulation in determining RH. Besides ischaemia and increased oxygen demand arterial hypoxia has also been reported to be an intensive stimulus for cardiac adenosine release [12, 14]. A significant parallelism has been demonstrated between coronary flow

and tissue adenosine content on one hand and the rate of adenosine release on the other from isolated guinea-pig hearts subjected to varying degrees of hypoxia [12]. In contrast to RH following mechanical occlusion, coronary vascular responses during severe hypoxia in the in situ heart are subjected not only to local, but also to neurohumoral factors activated by arterial oxygen deficiency [13].

The purpose of the present study was to investigate the quantitative aspects of the RH following coronary occlusion for 10 sec and of the hypoxic coronary vascular response during constant verapamil infusion in anaesthetized open-chest dogs. To distinguish between local and remote regulatory factors, various haemodynamic parameters were recorded simultaneously.

Materials and Methods

Mongrel dogs of either sex weighing 16 to 33 kg were anaesthetized by glucochloralose (100 mg. kg^{-1} body weight) with additional anaesthetic given as needed to maintain a constant level of anaesthesia. The animals were immobilized by flaxedyl (2 mg. kg^{-1}) and pulmonary ventilation was accomplished by a positive pressure respirator (Harvard) with room air enriched by 100% oxygen. Blood gases and pH were monitored (Radiometer Copenhagen, Type ABL 1) and they were kept within acceptable ranges. A femoral artery and vein were cannulated for continuous monitoring of mean arterial blood pressure (MABP) via a Statham P23HC pressure transducer, and for intravenous administration of fluids and drugs (Fig. 1). The heart was approached through a midsternal incision. An electromagnetic flow probe (Statham Sp2202) was placed around the ascending aorta to measure cardiac output (CO), and around the left anterior descending (LAD) coronary artery for monitoring coronary blood flow (CBF). Flow rates were measured by a Statham flow meter. The flow probes were calibrated in situ by cannulating the artery and pumping the dog's own blood through it at various known rates by a constant peristaltic pump (Harvard).

Conventional (lead II) and epicardial ECG were monitored and spontaneous heart rate was continuously recorded by an integrator fed by the R-wave signal of the ECG. Left ventricular pressure (LVP) was measured by a stainless steel needle inserted into the left ventricle and fixed by atraumatic sutures to the epicardium, via a Statham P23HC transducer. The dp/dt was continuously recorded by a derivative circuit fed by the ventricular pressure signal. All the above parameters were recorded on a 12-channel Grass Type 7D polygraph. The following parameters were calculated from the postocclusion reactive hyperaemic (RH) response (Fig. 2): basal conductance (BC = mean coronary blood flow/mean coronary perfusion pressure); maximal conductance (MC), peak conductance (PC = MC–BC); vascular reactivity (R = = PC/BC), the area of flow debt (A_1) and that of RH(A_2), and the ratio of them, i.e. the repayment (Rp = A_2/A_1). The magnitude of A_1 and A_2 was estimated by measuring the weight of the paper piece corresponding to flow debt (A_1) and postocclusion flow increases (A_2).

Experimental

Haemodynamic and coronary flow data were obtained in 8 animals 30 min after the start of recording when all parameters reached stable level. A 10-sec mechanical occlusion of LAD and 30-sec 100% N_2-inhalation were repeated twice (with 15 min intervals) to obtain control data for postocclusion reactive hyperaemia (RH) and hypoxic coronary vasodilatation. Verapamil (Verpamil®, Orion) was then given as a bolus injection of 0.2 mg. kg^{-1} intravenously, over a period of 2 to 3 min. This was followed by a constant infusion of the drug by a Harvard pump to deliver 0.01 mg. kg^{-1}. min^{-1} for a period of 60 min. LAD occlusion and N_2-inhalation were repeated twice after the effect of verapamil infusion had became stable ($\sim$30 min).

Statistical analysis: The means and standard error of the mean ($\bar{x} \pm$SEM) were calculated and the results were expressed in these terms. Statistical difference between means was estimated by Student's t-test.

Results

The original recording in Fig. 3 illustrates the effects of continuous verapamil infusion (0.01 mg. kg^{-1}. min^{-1}) on coronary blood flow and various cardiac and haemodynamic parameters during postocclusion (10 sec) reactive hyperaemia (RH) and arterial hypoxia (30 sec). Verapamil induced a transient increase in CBF and CO, and decreased MABP, HR, LVP and dp/dt max. RH was significantly depressed 30 min after the start of verapamil infusion but the increase of hypoxic coronary blood flow was not considerably influenced by the drug in the 50th min of perfusion. Table I demonstrates mean values of haemodynamic and cardiac variables before (control) and 15 min after verapamil infusion.

Cardiac output decreased slightly during verapamil infusion, however, the difference was not statistically significant. There was a significant ($p < 0.05$) drop of mean arterial blood pressure (MABP). Verapamil caused moderate reduction of total peripherial resistance (TPR). Heart rate (HR), dp/dt max and dp/dt min were significantly depressed by the drug. Verapamil failed to cause significant changes in mean coronary blood flow under the present experimental conditions.

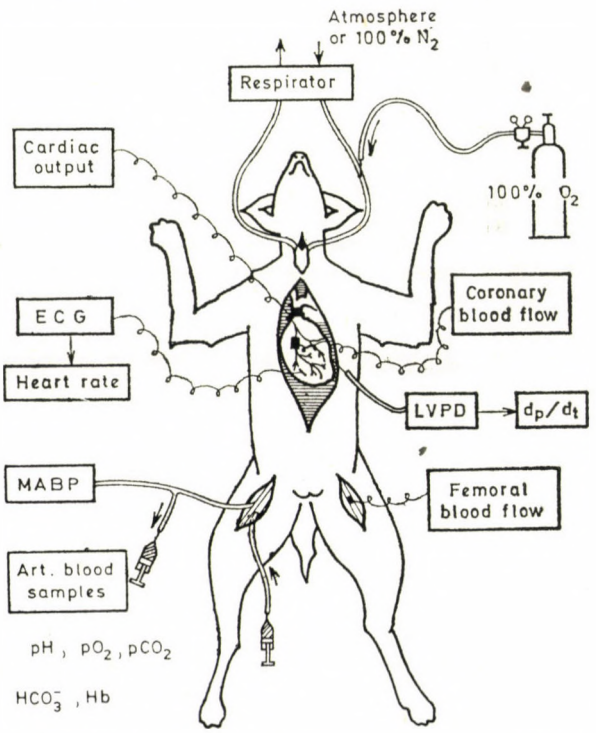

Fig. 1. Experimental model illustrating the measurement of various cardiac and haemodynamic parameters in open-chest dogs

Table I

*Haemodynamic changes during verapamil infusion**

Variable	Control	Verapamil	%	p**
C.O. (ml. min^{-1})	1463±133	1297±193	87.8±8.7	N.S.
MABP (mmHg)	110±7.6	86.2±5.1	78.6±5.0	0.05
TPR (mmHg. ml^{-1}. min^{-1})	0.074±0.007	0.066±0.008	87.9±5.9	N.S.
HR (beats. min^{-1})	183.5±6.0	154.9±8.2	84.6±4.6	0.05
dp/dt max (mmHg. sec^{-1})	2888±235	2212±217	79.2±7.8	0.05
dp/dt min (mmHg. sec^{-1})	2625±182	1969±213	76.9±8.9	0.05
CBF (ml. min^{-1})	27.8±3.8	26.7±5.7	90.0±10.9	N.S.

* mean ±SEM; n=8.
** N.S. = not significant.

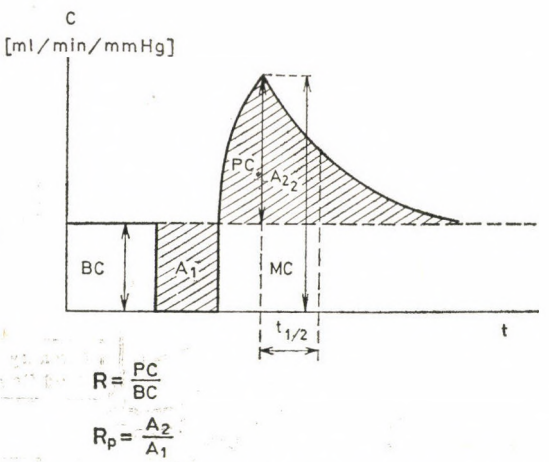

Fig. 2. Schematic diagrams illustrating various calculated parameters of postocclusion myocardial reactive hyperaemia

Myocardial reactive hyperaemia

The effect of verapamil on various parameters of RH (see also Fig. 2) is summarized in Table II. The decrease of basal coronary conductance (BC) was not statistically significant but all the obtained parameters of postocclusion reactive hyperaemia were significantly reduced by verapamil. Maximal conductance (MC) was diminished to 82% of control while peak conductance (PC) and vascular reactivity (R) were reduced by approximately 40%. The most pronounced inhibition was observed in the area of postocclusion blood flow

Table II

*Effect of verapamil on various parameters of reactive hyperaemia following 10-sec coronary occlusion**

Variable	Control	Verapamil	%	p**
BC	0.261±0.04	0.296±0.05	113.6±10.8	N.S.
MC	0.669±0.13	0.553±0.100	81.8± 7.8	0.05
PC	0.408±0.10	0.234±0.07	61.2±12.8	0.02
R	1.528±0.27	0.838±0.25	58.2±12.5	0.02
A_2	11.31 ±2.2	2.68 ±1.09	29.9±10.1	0.001
R_p	4.48 ±1.02	1.00 ±0.3	39.0±11.3	0.001

* mean ±SEM; n=8.
** N.S. = not significant.

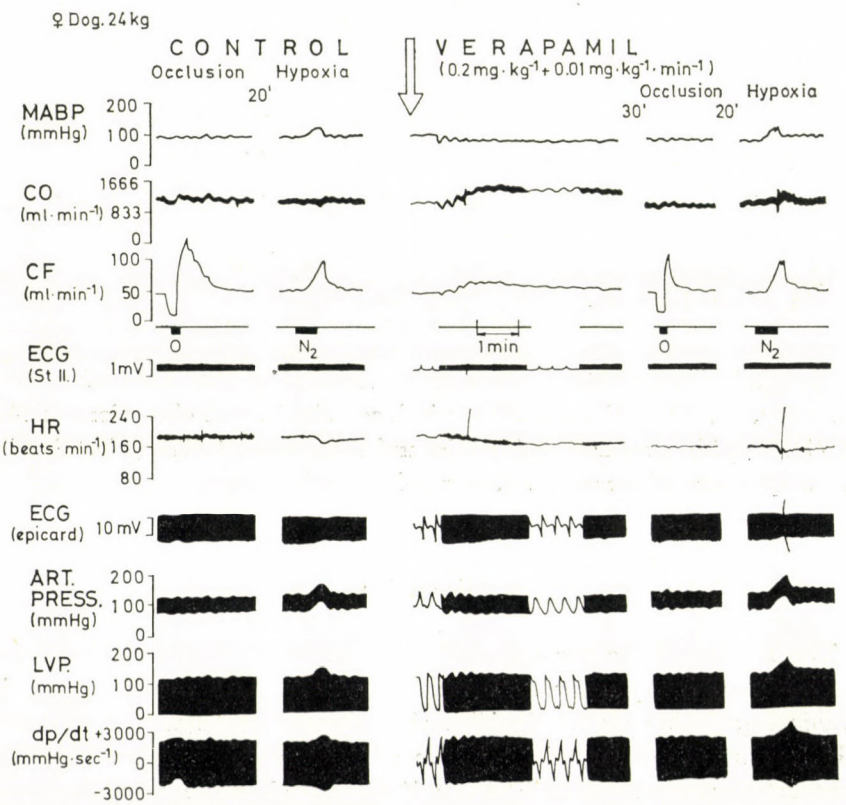

Fig. 3. Original recording demonstrating the effect of verapamil on various cardiac and haemodynamic parameters and on postocclusion myocardial reactive hyperaemia and N_2-induced arterial hypoxia in anaesthetized dog

change (A_2) and of flow repayment (Rp). The 4- to 5-fold overpayment of flow debt under control conditions was totally abolished by the Ca-antagonist: postocclusion increase of blood flow was equal to flow debt (i.e. repayment was exactly 1.00).

Arterial hypoxia

In sharp contrast to the significant suppression of postocclusion reactive hyperaemia, 0.01 mg. kg^{-1}. min^{-1} verapamil infusion did not influence maximal conductance, peak conductance and vascular reactivity during hypoxic coronary vasodilation induced by 30 sec 100% N_2-inhalation (Table III). While the 10-sec coronary occlusion caused no significant change in the

Table III

Effect of verapamil on variables of hypoxic coronary vasodilatation

Variables	Control	Verapamil	%	p**
BC	0.216±0.034	0.226±0.025	111.5± 9.1	N.S.
MC	0.316±0.038	0.326±0.025	112.5± 9.6	N.S.
PC	0.100±0.016	0.100±0.027	104.6±23.4	N.S.
R	0.553±0.134	0.468±0.109	81.4±20.1	N.S.

* mean ±SEM; n=8.
** N.S. = not significant.

haemodynamic and cardiac parameters, indicating the activation of local factors only, N_2-inhalation for 30 sec induced significant elevations of MABP, CO, LVDP, dp/dt max and dp/dt min and reduction of HR (Fig. 3) pointing to the activation of remote, i.e. neuro-humoral factors.

Discussion

The results of the present study have shown that characteristic parameters of reactive hyperaemia of the coronary vessels following a 10-sec mechanical occlusion can be significantly reduced, although to various extent, by the specific Ca-antagonist verapamil in open-chest anaesthetized dogs. In contrast, augmentation of CBF by 100% N_2-inhalation for 30 sec was not significantly influenced by the drug under the present experimental conditions.

The possible mechanism by which verapamil might have reduced RH are best considered in relation to its pharmacological properties. There are numerous data available to suggest that the primary action of the drug is selective inhibition of the movement of the calcium ion across the membrane of excitable tissues [5]. Since calcium ions have been reported to play a key role in both excitation-contraction coupling and pacemaker function in the heart this action of the drug has been postulated to be responsible for its negative inotropic and chronotropic actions in cardiac muscle [5]. The present finding of significantly reduced spontaneous heart rate and left ventricular dp/dt max during verapamil infusion are in good agreement with these previous reports.

In addition, it has been shown that under the influence of Ca-antagonist compounds the splitting of ATP, the contractile energy expenditure and oxygen requirement of the beating heart are lowered [5]. Although no attempt were made in the present study to measure either ATP splitting or O_2 uptake in the myocardium, the finding that verapamil significantly reduced all major determinants of cardiac O_2 consumption [15] (i.e. MABP (afterload), HR, and dp/dt max), it can be assumed that, in parallel with cardiac function, myocardial energy and O_2-requirements were significantly depressed as well.

If we accept the concept that RH is caused by the accumulation of vasodilatator metabolite(s) (adenosine) during ischaemia [10], it is clear that a decline of metabolic rate in the presence of verapamil would reduce the production and consequent accumulation of metabolites and the magnitude of RH. However, other factors may also be involved. Increased rate of adenosine washout, degradation or reuptake may all contribute to the depression of RH. Altough these processes are not likely to be altered by verapamil, they should be checked in the future.

Two further alternative mechanisms have been suggested to be responsible (at least in part) for RH, which may also be influenced by verapamil. The first is based on the hypothesis that RH is due to the metabolic consequences of hypoxia within the coronary vessels themselves [1]. Our results could have been explained also by this concept if we assume that similar to myocardial cells verapamil reduces the oxygen requirement of vascular smooth muscle cells as well.

Reactive hyperaemia has also been atributed to myogenic relaxation of the coronary smooth muscle cells during the period of arterial occlusion. After release of the occlusion myogenic tone is though to be stimulated by the distending force exerted by increased intraluminal pressure. This mechanism called autoregulation probably reflects the contractile state of the vascular smooth muscle prior to the change in pressure [2, 10]. Verapamil was reported to inhibit E–C coupling and pacemaker activity of isolated coronary arteries and, as a result, acts as a powerful coronary vascular smooth muscle relaxant

[5]. It has been shown that smooth muscle contraction following stretch, similar to that seen during autoregulation, is critically dependent on inward Ca^{2+}-movement across the smooth muscle cell membrane. Thus verapamil may reduce RH by inhibiting this myogenic mechanism.

The possibility also exists that the response of coronary vessels to a given intensity of stimulus (like adenosine) may be altered by verapamil. This possibility is confirmed by a previous finding that adenosine-induced coronary vasodilatation in the dog is antagonized by lanthanium [7]. Studies on isolated helical coronary artery strips revealed that adenosine, similarly to verapamil, inhibits Ca^{2+}-influx into vascular smooth muscle strips [6]. A possible explanation of this paradox finding is based on the dual role of Ca^{2+} in vascular smooth muscle cells, i.e. activation of contraction and membrane stabilization [4]. It is possible that adenosine enhances the binding of Ca^{2+} on smooth muscle membrane, resulting in a decreased excitability and relaxation, which is abolished by verapamil through reduction of the amount of Ca^{2+} adjacent to the cell membrane. These hypotheses should be tested experimentally in the future.

Since postocclusion reactive hyperaemia in the heart is a complex response [10], one may speculate that verapamil eventually antagonizes one (or more) factors while other mechanism(s) remain intact. This hypothesis may be empirically substantiated by the finding that the significant overpayment (448%) of flow debt under control conditions was reduced to a mean of 100% (i.e. no overpayment at all) by the drug.

The possibility exists that verapamil antagonizes factor(s) that are, at least hypothetically, responsible for the disproportional overpayment of flow debt [10] and thus the drug may be a valuable tool to study the possible cause(s) of overpayment of both flow and oxygen debt [10].

It is likely that hypoxia-induced increase of coronary perfusion pressure (MABP) and aortic flow, and the possible coronary dilating action of released catecholamines, counterbalance the verapamil induced inhibition of effects of local factors (i.e. hypoxia and/or increased adenosine release) induced by cardiac activity under the present experimental conditions. The present finding is consistent with previous data that major determinants of CBF changes during arterial oxygen deficiency are the extracardiac neuro-humoral factors activated by hypoxia [13].

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РЕЗЮМЕ

ИЗОЛИРОВАНИЕ МИОЗИНОВ ИЗ ЧЕЛОВЕЧЕСКОГО МОЗГА, ИХ СВОЙСТВА И СОДЕРЖАНИЕ В НИХ ФОСФОРА

Ш. ФАЗЕКАШ, И. ОВАРИ, Ф. ХОРВАТ, В. СЕКЕШШИ-ГЕРМАНН и П. ЮХАС

В наших экспериментах мы изолировали KCl- и NaCl-миозин из разных отделов центральной нервной системы человека. На протяжении всех опытов содержание фосфора и липидов было выше в NaCl-миозине, чем в препаратах с хлористым калием. Однако, концентрация фосфора и липидов в KCl- и NaCl-миозинах тоже было выше, чем в миозинах скелетных мышц.

Когда мы изолировали образцы миозинов на колонке 4B Сефарозы с помощью техники молекулярной фильтрации, то обнаружили, что миозины мозжечка и коры головного мозга составлены из двух фракций и различной молярной массой, тогда как миозины мозгового ствола и спинного мозга выделяются только как простой и единственный пик.

Миозиновые препараты располагают Ca-АТРазной активностью и эту активность усиливает актин поперечно-полосатой мышцы кролика.

Филаментарные системы и агрегаты препаратов миозина обладают способностью к развитию, что можно показать на электронномикроскопических снимках.

Миозиновые препараты центральной нервной сисмы могли фосфорилировать, различные миозины в разной степени, присутствие серотонина (или отсутствие в инкубационной среде) оказывало влияние на прием препаратами фосфата.

Изучение кинетики показало, что насыщение фосфатом миозинов ствола мозга, мозжечка и коры головного мозга зависит от продолжительности инкубирования и от концентрации АТР в инкубационной среде.

Щелочные гидролизаты безлипидных миозинов головного мозга человека содержат фосфаты аминокислот (P—Arg, P—Lys и P—His), количество и пропорция которых отличаются, в зависимости от места происхождения миозиновых препаратов. Под влиянием фосфорилирующей смеси в церебральном миозине возрастает только количество P—Arg, в препаратах ствола мозга только P—Arg и P—His, в мозжечковых миозинах P—Arg, P—His и количество не отождествленного фосфорилированного компонента.

ВЛИЯНИЕ ИНДОМЕТАЦИНА НА ФУНКЦИЮ ПОЧЕК НА НАРКОТИЗИРОВАННЫХ СОБАКАХ ПРИ ПОВЫШЕННОМ ОПЕРАЦИОННОМ СТРЕССЕ

Д. КЭВЕР, КАТАЛИН СЕМЕРЕДИ, ХИЛДА ТОСТ

Функция почек наркотизированных собак изучалось при лёгком и тяжёлом операционном стрессе, когда интравенозным введением раствора Рингера вызывалось увеличение внеклеточного объёма меньшей или большей степени, далее у собак, синтез простагландина которых был ингибирован интравенозно 4 мг/кг индометацином.

Наблюдалось, что гемодинамические параметры ($C_{РАН}$, $C_{инулин}$) при тяжёлом операционном стрессе соответствуют параметрам, зарегистрированные при легком операционном стрессе и также не отличается выделение воды и натрия почками при незначительном увеличении внеклеточного объёма.

При увеличении экстрацелюлярного объёма, у животных, находившихся в более тяжёлом операционном стрессе, усиление выделения натрия значительно, усиление выделения воды в меньшей степени отстают по сравнению с изменением, наблюдаемое на животных при легком операционном стрессе.

Индометацин уменьшал выделение мочи и натрия почками в обоих группах при неизменной клубочковой филтрации. C_{PAH} снизилась на 20 – 25% при легком, и на 35 – 40% при тяжёлом операционном стрессе. В последней группе количество протекающей через почки крови (RBF_{dir}) снижалось на 40% после введения индометацина по сравнению с контрольным периодом опыта. При легком операционном стрессе степень повышающегося выделения натрия, наблюдаемое во время увеличенного внеклеточного объёма после введения индометацина, была одинакова с величиной контрольной группы, но мочеотделение и выделение натрия не усиливались.

Интравенозное введение одинаковой дозы индометацина вызывает более выраженное снижение выделения натрия и воды при тяжёлом операционном стрессе. Объясняя этот феномен придаётся значение гемодинамическим изменениям почек. Так, более выраженное уменьшение кровянного тока почек значительно усиливает реабсорбцию воды и натрия на проксимальных канальцах. В наших опытах нет необходимости предполагать специфического действия простагландинов на функции тубулярных клеток.

ИССЛЕДОВАНИЯ АНТАГОНИЗМА ИНДОМЕТАЦИНА И БРАДИКИНИНА НА НАРКОТИЗИРОВАННЫХ СОБАКАХ

ХИЛДА ТОСТ, П. КЭВЕР, КАТАЛИН СЕМЕРЕДИ

С целью изучения влияния брадикинина на функцию почек, в том числе и в зависимости от простагландина, проводили инфузию брадикинина в количестве 0,05 мкг/кг в мин. в левую почечную артерию подопытных животных, синтез простагландина которых был ингибирован индометацином.

Интравенозная инфузия индометацина в количестве 0,1 мг/кг в мин. вызывала значительное уменьшение выделения натрия и мочи у наркотизированных животных. Сопротивление почечных сосудов увеличивалось, количество крови, протекающей через почки (RBF_{dir}) снижалось на 30%.

Когда вводился брадикинин в левую почечную артерию при продолжительной интравенозной инфузии индометацина, тогда количество протекающей крови через левую почку и выделение мочи и натрия левой почкой возвращались на уровень контроля, а в правой, не инфундированной почке оставались и в дальнейшем низким.

Результаты указывают на то, что брадикинин увеличивает количество крови, протекающей через почки независимо от простагландинов.

В результате инфузии индометацином в каждом случае уменьшается RBF, которое и обясняет наблюдаемое уменьшение выделения мочи и натрия под влиянием индометацина.

Действие брадикинина на сосудистую систему почек по видимому непосредственное, а влияние, оказываемое на выделение мочи и натрия является, как предполагается, посредственным.

Так как индометацин не тормозит диуретическое и натриуретическое действие брадикинина, предполагается, что в этом простагландины не играют существенную роль

ВЛИЯНИЕ АНТИДИУРЕТИЧЕСКОГО ГОРМОНА НА РАСПРЕДЕЛЕНИЕ ВНУТРИПОЧЕЧНЫХ МИКРОСФЕР

Г. ФЕЙЕШ-ТОТ, Т. ЗАХАЙСКИ, Й. ФИЛЕП

Целью настоящих экспериментов являлась изучение влияния антидиуретического гормона на распределение внутрипочечного кровообращения у наркотизированных крыс с применением техники микросфер. Введением аргинин-вазопрессина (ВП) в зависимости от доз (3,6,60 mU на кг в час) вызывалось антидиурез. Клубочковая фильтрация усиливалась только от введения ВП 60mU/кг в час, а при низких доз не изменились. Инфузия ВП в маленьких дозах не приводила к изменению распределения внутрикорковых микросфер, но

под влиянием инфузии ВП 60mU/кг в час соотношение внешнего и внутреннего коркового кровотока стало меньше.

Результаты указывают на то, что физиологический уровень ВП не изменяет распределение внутриренального кровотока и антидиуретическое действие ВП не зависит от гемодинамических изменений почек.

ФАРМАКОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ФУНКЦИОНАЛЬНОГО СОСТОЯНИЯ В СТЕНОЗИРОВАННОМ КОРОНАРНОМ СОСУДЕ

В. КЕЙКЕШИ и А. ЮХАС-НАДЬ

В экспериментах на собаках с открытой грудной клеткой, находящихся под пентобарбиталовым наркозом, авторы изучали действие механического сужения одной из ветвей большого коронарного сосуда на способность к авторегулированию, относящихся к тому сосуду маленьких сосудов сопротивления.

Коронарный кровоток определяли электромагнитным измерителем кровотока. Ступенчатое механическое сужение большой ветви постепенно уменьшало вызванное аденозином расширение коронарного сосуда, тогда как уровень покоя коронарного кровотока оставался неизменным. В этой фазе верапамил, располагающий собственным вазодилататорным действием, но сильно блокирующий эффект аденозина/будучи введен внутривенно, в дозе 0,2 мг/кг/, уменьшал коронарный кровоток и повышал сосудистое сопротивление венечных сосудов. Подобные результаты получили также при непосредственном введении этих препаратов в анастомоз, осуществленный между сонной артерией и общей левой коронарной артерией.

Результаты экспериментов показывают, что

1. аденозин воздействует на те же самые коронарные сегменты, которые принимают участие в компенсирующей авторегуляции;
2. в случае коронарного стеноза критического уровня верапамил увеличивает сосудистое сопротивление коронарных сосудов, через ингибирование «внутреннего» аденозинового действия;
3. функциональное состояние суженных коронарных сосудов можно оценить помощью описанного фармакологического теста.

ВЫЗВАННАЯ НИКЕЛЕМ ВАЗОКОНСТРИКЦИЯ ИЗОЛИРОВАННОЙ КОРОНАРНОЙ АРТЕРИИ СОБАКИ НА ОСНОВЕ МЕХАНИЗМА ТОНИЧЕСКОГО АКТИВИРОВАНИЯ ИОНОВ КАЛЬЦИЯ

Г. РУБАНИ, Л. КАЛАБАИ, Т. ПАТАКИ и К. ХАЙДУ

Хлористый никель в низкой концентрации (1 мкМ) вызывает увеличение изометрического напряжения в полоске изолированной коронарной артерии собаки. Эффект Ni^{2+} является функцией концентрации внеклеточного кальция (0—5 мМ). Верапамил (10^{-6}—10^{-3} М) не подавляя эффект, вызываемый ионами никеля, однако натропруссид натрия (10 М) эффективно антагонизировал сокращение коронарной артерии, вызванное Ni^{2+}.

Результаты экспериментов показывают, что повышение изометрического напряжения изолированной коронарной артерии собаки под действием ионов никеля связано с вхождением в клетку ионов кальция, что осуществляется посредством активации Ca^{2+} через Т-систему.

РОЛЬ АДРЕНЕРГИЧЕСКИХ РЕЦЕПТОРОВ В ВЫЗВАННОЙ ИОНАМИ НИКЕЛЯ КОРОНАРНОЙ ВАЗОКОНСТРУКЦИИ

Г. РУБАНИ, К. ХАЙДУ, Т. ПАТАКИ и М. БАКОШ

Авторы настоящей статьи на изолированной коронарной артерии собаки и изолированном перфузируемом сердце крысы изучали возможную роль адренергических рецепторов в механизме коронарного стеноза, вызванного ионами никеля. Эксперименты, выполненные на двух моделях, привели к нижеследующим однозначным выводам:

(1) ингибирование феноксибензамином или фентоламином альфа-адренергических рецепторов лишь частично подавляло действие ионов никеля;

(2) пропранолол, блокирующий бета-адренергические рецепторы, полностью предотвращал действие никеля;

(3) Ni^{2+} (1 мкМ) достоверно тормозил расширение коронарных сосудов, вызванное раздражением (изопротеренол) бета-рецепторов.

Экспериментальные результаты показывают, что роль альфа-рецепторов ничтожна, однако можно предположить, что действие ионов никеля осуществляется посредством бета-рецепторного механизма. Ni^{2+} изменяет реактивность бета-рецепторов коронарных сосудов, что указывает на участие микроэлемента в модулировании адренергических механизмов.

КОРОНАРНАЯ ВАЗОКОНСТРИКЦИЯ, ВЫЗВАННАЯ НИКЕЛЕМ, ЗАВИСИТ ОТ АКТИВНОСТИ ЭЛЕКТРОГЕННОГО НАТРИЕВО—КАЛИЕВОГО НАСОСА

Г. РУБАНИ, М. БАКОШ, К. ХАЙДУ и Т. ПАТАКИ

Целью настоящих экспериментов являлось исследование взаимодействия ионов никеля (Ni^{2+}) и Na—K—АТРазы на изолированном перфузируемом сердце крысы и коронарной артерии собаки.

Особенности повышения изометрического напряжения, вызванного ионами никеля в опытах с изолированной коронарной артерией, согласуются с механическим ответом, вызванным ингибированием Na—K—АТРазы. Ингибирование активности насоса оуабаином (10^{-4} М) и лишенным K^+ раствором Кребса затормаживало проявление эффекта никеля в обоих препаратах. Результаты экспериментов указывают на то, что, в ходе сужения коронарных артерий, вызванного ионами никеля, возникает $Na^+—K^+$ обмен. Необходимо продолжить опыты, чтобы выяснить, непосредственно ли влияет ион никеля на энзим и является ли его действие только функцией ионного градиента, поддерживаемого электрогенным Na—K насосом.

ВЛИЯНИЕ КАТИОНОВ (Ni, Zn, Fe, Co, Hg, Cu) И МЕТАВАНАДАТА НА КОРОНАРНОЕ СОСУДИСТОЕ СОПРОТИВЛЕНИЕ В ИЗОЛИРОВАННОМ ПЕРФУЗИРУЕМОМ СЕРДЦЕ КРЫСЫ

М. БАКОШ и Г. РУБАНИ

Авторы исследовали влияние, оказываемое двувалентными катионами и метаванадатом на сопротивление коронарных сосудов в изолированном перфузируемом сердце крысы. На основании активности элементы расположились в следующем порядке (в скобках приводится концентрация катиона в мкМ, вызывающая 50% сужение коронарных сосудов): Ni^{2+} (0,03) > Co^{2+} (0,1) > Hg^{2+} (0,16) > VO^-_3 (0,2) > Cu^{2+} (15) > Zn^{2+} (50). Ионы железа (Fe^{2+}) и меди (Cu^{2+}) оказались неэффективными в примененных концентрациях (0,01—100 мкМ). Приведенный ряд активности отличается от любого другого ряда, основанного на физико-химических свойствах элементов, и это показывает, что коронарный вазоконстрикторный эффект катионов нельзя объяснить исключительно взаимодействием между мембраной и ионом металла. Феноксибензамин полностью предотвращал действие ртути (в противоположность действию никеля), и это доказывает, что вазоконстрикторный эффект на венечные сосуды Hg^{2+} осуществляет через альфа-адренергический механизм. В противоположность катионам никеля и ртути, действие VO^-_3 не ингибировалось верапамилом. Последнее наблюдение указывает на то, что ванадат оказывает свой эффект посредством мобилизации внутриклеточного кальция.

ВЛИЯНИЕ ИОНОВ НИКЕЛЯ НА СПОНТАННЫЕ, ВЫЗВАННЫЕ ЭЛЕКТРИЧЕСКИМ РАЗДРАЖЕНИЕМ И СТИМУЛИРОВАННЫЕ НОРАДРЕНАЛИНОМ ИЗОМЕРИЧЕСКИЕ СОКРАЩЕНИЯ ИЗОЛИРОВАННОЙ ПОРТАЛЬНОЙ ВЕНЫ КРЫСЫ

Г. РУБАНИ и И. ИНОВАИ

Мы изучали влияние ионов никеля (1—10 мкМ) на спонтанную сократительную активность препарата изолированной портальной вены крысы, а также на усиление изометрического напряжения, вызванного электрическим пространственным раздражением (80 В/5 см, 1 мсек, 1—32 Гц) или примененным наружно норадреналином.

Ni^{2+} тормозил спонтанное повышение напряжения, снижал основной тонус, но увеличивал частоту спонтанных сокращений. Ионы инкеля гораздо более выраженно тормозили повышение напряжения, вызванное электрическим раздражением, чем норадреналином, что указывает как на пресинаптическое (освобождение норадреналина из нервных окончаний), так и на постсинаптическое действие ионов никеля на этом сосудистом препарате.

ТОРМОЖЕНИЕ РЕАКТИВНОЙ ГИПЕРЕМИИ МИОКАРДА НЕ ГИПОКСИЧЕСКОЙ КОРОНАРНОЙ ВАЗОКОНСТРИКЦИЕЙ, А ПОСТОЯННОЙ ИНФУЗИЕЙ ВЕРАПАМИЛА В СЕРДЦЕ IN SITU

А. КОЛЛЕР, Г. РУБАНИ, Л. ЛИГЕТИ, А. Г. Б. КОВАЧ

Изучено изменение характерных параметров реактивной гиперемии миокарда и гипоксической ответной реакции коронарных сосудов, вызванное механическим сжатием в течение 10 сек. под действием постоянной инфузии верапамила (0,01 мг/кг в мин.) в опытах на собаках с открытой грудной клеткой.

Верапамил со специфическим калциевым антагонизмом достоверно уменьшил реактивную гиперемию, но увеличение кровотока в коронарных сосудах, вызванное ингаляцией N_2 в течение 30 сек. было незначительно. Экспериментальные данные указывают на то, что верапамил также уменьшает потребность кислорода гладких мышц сосудов, как и клеток миокарда. Предполагается, что некоторые факторы, ответственные за значительное расплачивание кровоточного долга в последствие окклюзии, элиминируется верапамилом.

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Physiologia—Pathophysiologia

EFFECT OF ISOPROTERENOL ON RENAL FUNCTION

By

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The effects on renal function of isoproterenol and vasopressin were compared in conscious hydrated dogs.

In response to isoproterenol infusion (12 μg/kg/h) urine flow, sodium and potassium excretion, free water clearance dropped markedly whereas glomerular filtration rate, blood flow, and the distribution of cortical blood flow remained practically unaffected.

Vasopressin infusion (2 mU/kg/h) produced an antidiuresis comparable to that after isoproterenol infusion, but sodium and potassium excretion was considerably enhanced.

Since isoproterenol and vasopressin are known to exert opposing effects on ion excretion, it is suggested that the increased secretion of vasopressin cannot play an exclusive role in the development of changes of renal function induced by isoproterenol.

Infusion of beta-adrenergic isoproterenol into the renal artery produces an increase in renal blood flow [14] and exerts a moderate diuretic effect [8]. On the other hand, intravenous infusion of the drug causes a marked decrease in urine flow [6, 11, 12]. The mechanism of this antidiuretic effect has not been clarified. In some studies the antidiuresis was accompanied by a decrease in sodium excretion [9, 10] while in others, the electrolyte excretion were unaltered [2]. Little is known about the renal haemodynamic action of isoproterenol administered intravenously. The drug has been shown to enhance renal blood flow in the anaesthetized dog [9] and to increase the clearance of PAH in man [3]. A widely accepted view attributes an important role to vasopressin release mediated by beta-adrenergic stimulation [16]. We have, therefore, compared the renal effects of isoproterenol and vasopressin infusion producing an antidiuretic action of the same magnitude. In addition, we have studied the effect of intravenously administered isoproterenol on water and electrolyte excretion and intrarenal haemodynamics.

Methods

Experiments were performed on conscious female mongrel dogs weighing 10–21 kg. In one group of the animals two weeks before the experiments perineotomy was made under pentobarbital anaesthesia and a catheter was implanted into the right carotid artery up to the left ventricle, the location was confirmed by pressure tracing. Food was withheld 18 h before the experiments but animals had free access to water. Immediately before beginning the experiment under local anaesthesia a catheter was inserted into the abdominal aorta via the right

saphenous artery for monitoring blood pressure, and measuring reference blood flow, and another catheter was inserted into the saphenous vein for the infusions. Then the dogs were hydrated with 3.5% body weight tap water via a gastric tube. After 30 min the hydration was repeated. Water loss by urine was substituted in fractions of 200 ml. After an equilibration period of at least 2 h two control urine collecting periods were obtained. Urine was collected through a self-retaining bladder catheter. Mean arterial blood pressure was recorded by an electric manometer. Cardiac output, renal blood flow and renal cortical blood flow distribution were determined with use of radioactive microspheres tagged with ^{85}Sr or ^{141}Ce (13.2 $\pm$ 0.9 μm and 13.9 $\pm$ 1.0 μm in size respectively, 3M Corp.) injected into the left ventricle through the carotid catheter. Fifteen min after the first microsphere injection isoproterenol (Isuprel, Winthrop) at a dose of 12 μg/kg/h was added to the normal saline infused continuously from the beginning of the experiment at a rate of 15 ml/h. One hour after the onset of the infusion the second microsphere injection was given. Both microsphere injections were preceded and followed by 15-min urine collection periods. Arterial blood samples were obtained at the midpoint of these periods. Another group of dogs was subjected to similar experiments except that after control periods an infusion of vasopressin (synthetic arginine vasopressin, grade V, Sigma) was started at a dose of 2 mU/kg/h. In these experiments no microsphere was given. Inulin was measured by the method of FÜHR [7]. Urine and plasma samples were analyzed for sodium and potassium by flame photometry. Osmotic concentration was determined by freezing-point depression.

Values obtained in the two control and two experimental periods were averaged. The data were statistically evaluated by Student's t test for paired values.

Results

Effect of isoproterenol

The haemodynamic parameters are given in Table I. The heart rate was accelerated from 129 $\pm$ 9/min to 167 $\pm$ 7/min by isoproterenol and this was accompanied by a marked increase in cardiac output. Arterial blood pressure was depressed moderately but significantly. Total peripherial resistance decreased considerably. The changes in total renal resistance and renal blood flow were not statistically significant. Glomerular filtration rate and filtration fraction were practically unaffected.

Table I

Effect of isoproterenol on systemic and renal haemodynamics

n = 8	MAP mmHg	CO ml/min	TPR $\frac{\text{mmHg}}{\text{ml/s}}$	RBF ml/min	TRR $\frac{\text{mmHg}}{\text{ml/s}}$	GFR ml/min	FF
Control	110 $\pm$ 4	192 $\pm$ 22	38.4 $\pm$ 5.6	415 $\pm$ 25	1.64 $\pm$ 0.14	87 $\pm$ 7	0.35 $\pm$ 0.02
Isoproterenol	99 $\pm$ 4	309 $\pm$ 16	19.6 $\pm$ 1.3	493 $\pm$ 30	1.43 $\pm$ 0.11	95 $\pm$ 10	0.39 $\pm$ 0.05
$p <$	0.01	0.001	0.01	NS	NS	NS	NS

Data are means $\pm$ SE. Abbreviations: n, number of observations; MAP, mean arterial pressure; CO, cardiac output; TPR, total peripheral resistance; RBF, renal blood flow; TRR, total renal resistance; GFR, glomerular filtration rate; FF, filtration fraction. RBF and GFR are calculated for 100 g kidney wt. TPR, TRR and CO are referred to 1 kg body weight. NS, not significant.

Table II

Effect of isoproterenol on water and electrolyte excretion

n = 7	V ml/min	U_{osmol} μosmol/ml	C_{H_2O} ml/min	$U_{Na}V$ μeq/min	U_KV μeq/min
Control	9.2 ± 1.0	62 ± 3	7.2 ± 0.8	82 ± 20	54 ± 6
Isoproterenol	4.4 ± 0.8	102 ± 15	2.9 ± 0.7	34 ± 10	34 ± 6
$p <$	0.01	0.05	0.01	0.05	0.05

Values are means ± SE. V, urine flow rate; U_{osmol}, urinary osmotic concentration; C_{H_2O}, free water clearance; $U_{Na}V$, urinary sodium excretion; U_KV, urinary potassium excretion; n, number of observations. V, C_{H_2O}, $U_{Na}V$ and U_KV are referred to 100 g kidney wt.

Values for water and electrolyte excretion are given in Table II. A marked reduction of the urine flow was accompanied by the increase of the urinary osmotic concentration leading to a decrease of the free water clearance. During this antidiuresis, sodium and potassium excretions were considerably decreased.

Data for intracortical blood flow distribution are shown in Table III. Zonal perfusion rates and thus the intracortical blood flow distribution were not affected by isoproterenol.

Table III

Effect of isoproterenol on intracortical blood flow distribution

n = 8	Zonal cortical blood flow ml · min^{-1} · g^{-1}			
	Z_1	Z_2	Z_3	Z_4
Control	7.80 ± 0.35	6.51 ± 0.53	4.44 ± 0.44	2.40 ± 0.29
Isoproterenol	7.54 ± 0.70	8.13 ± 0.85	5.10 ± 0.49	2.24 ± 0.14
$p <$	NS	NS	NS	NS
n = 8	Percent distribution of intracortical blood flow			
	Z_1	Z_2	Z_3	Z_4
Control	37 ± 2.4	31 ± 1.2	21 ± 1.0	11 ± 0.8
Isoproterenol	33 ± 1.5	35 ± 1.1	22 ± 1.5	10 ± 0.6
$p <$	NS	NS	NS	NS

Values are means ± SE. Abbreviations: Z_1—Z_4, represent cortical zones Z_1 being the outermost and Z_4 the innermost zone.

Table IV

Effect of vasopressin on renal function

n = 6	GFR ml/min	V ml/min	U_{osmol} μosmol/ml	C_{H_2O} ml/min	$U_{Na}V$ μeq/min	U_KV μeq/min
Control	50 ± 4.0	8.8 ± 1.2	37 ± 1	7.6 ± 1.0	93.8 ± 24.4	10.9 ± 2.2
Vasopressin	50 ± 4.2	5.0 ± 0.9	112 ± 15	3.2 ± 0.8	169.3 ± 29.8	20.6 ± 4.3
$p <$	NS	0.01	0.001	0.001	0.05	0.05

Values are means ± SE. Abbreviations: see legend for Tables I and II. GFR, V, C_{H_2O}, $U_{Na}V$ and U_KV are referred to 100 g kidney wt.

Effect of vasopressin

Values for renal effects of vasopressin are given in Table IV. Glomerular filtration rate was not changed. The reduction of urine flow, the increase of the urinary osmotic concentration and the decrease of the free water clearance were similar to changes that occurred during isoproterenol infusion. The sodium and potassium excretions were, however, considerably enhanced by vasopressin.

Discussion

Isoproterenol infused intravenously produces antidiuresis [2, 3, 10, 13, 15, 16] while its administration into the renal artery causes diuresis [8, 16]. In the former effect the concomitant release of vasopressin seems to play an important role. Isoproterenol failed to elicit antidiuresis in hypophysectomized dogs [16]. Isoproterenol infused to patients with untreated diabetes insipidus was ineffective in a dose which is known to produce antidiuresis in water-loaded volunteers [3]. Recently it was found that the plasma vasopressin concentration was elevated in conscious dogs after subcutaneous injection of isoproterenol [15].

Isoproterenol infusion into the carotid artery did not alter the urine flow rate in anaesthetised dogs [2], therefore a direct action on the neurohypophysis seems unlikely. Some indirect effect producing vasopressin release might be probable. The fact that carotid sinus and aortic arch denervation prevents the antidiuretic action of isoproterenol indicates the importance of the baroreceptors [2]. The administration of the angiotensin II-receptor antagonist (Sar^2, Ala^8)-angiotensin II into the third ventricle of the brain in conscious dogs could prevent the isoproterenol-induced vasopressin release [15] suggesting the involvement of the renin-angiotensin system.

According to some studies, isoproterenol-induced antidiuresis may occur in certain instances without vasopressin release. Namely, when antidiuresis

was produced by the intravenous infusion of isoproterenol in conscious dogs, no increase in the antidiuretic activity of the jugular plasma could be observed [10]. In Brattleboro rats intravenous isoproterenol caused antidiuresis [13].

The question thus arises whether the isoproterenol induced changes in renal function can be attributed exclusively to vasopressin release. Comparison of the electrolyte excretion might help to solve the problem. It is well known that vasopressin increases sodium and potassium excretion [cf. 4], but the scant reports on the effect of isoproterenol administered intravenously on electrolyte excretion are controversial, with both decrease and increase [9, 10] being reported. However, BERL [2] has found no change in urinary sodium and potassium excretion.

In order further to elucidate the problem, one group of conscious water-loaded dogs were infused intravenously with isoproterenol whereas the other group was treated with vasopressin at a dose producing antidiuresis of the same magnitude as did the isoproterenol. After vasopressin treatment the diuresis was associated with an increased sodium and potassium excretion as had been expected whereas in response to isoproterenol the antidiuresis was accompanied by a marked reduction in sodium and potassium excretion. Thus, even if vasopressin does play a role in the renal effect of the intravenously administrated isoproterenol, under the present experimental conditions it could not be exclusive.

The intracortical distribution of radioactive microspheres after the intravenous infusion of isoproterenol has also been analysed. The microsphere technique has recently been strongly criticized (cf. 1). In the knowledge of the microsphere distribution pattern one cannot draw conclusions concerning the intracortical blood flow distribution without risking serious mistakes. This method should be restricted to those instances when the unchanged distribution of microspheres is associated with an unchanged total renal resistance indicating an unchanged blood flow distribution. Since in the present study neither glomerular filtration rate and renal blood flow nor the microsphere distribution pattern changed significantly, alterations in water and electrolyte excretion seem to be independent of changes in renal haemodynamics.

Our results appear to suggest that under the present experimental conditions: 1. The isoproterenol-induced antidiuresis is associated with reduced sodium and potassium excretion. 2. The responses developed cannot be attributed exclusively to vasopressin release. 3. These effects of isoproterenol on renal function seems to be independent of changes in renal haemodynamics.

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ELEVATION OF THERMOREGULATORY VASODILATATION THRESHOLD IN THE RAT AFTER CAPSAICIN TREATMENT*

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In control and capsaicin-treated (300 mg/kg) rats tail skin vasodilatation was studied while the body temperature was raised to 38 °C, 39 °C or 40 °C and held at these levels. In the capsaicin-treated rats, at 38 °C vasodilatation was weaker than in the controls but at temperatures of 39 °C and 40 °C a delayed increase in tail vasodilatation occurred to the level observed in the controls. It is concluded that the threshold of vasodilatation response to heat is elevated after capsaicin treatment.

Repeated capsaicin treatment results in a severe impairment of rat thermolysis: the animals become hyperthermic in a warm environment [7, 8], while saliva secretion and grooming decrease [2, 10]. All authors [6, 10, 11, 12, 13] but one [2] reported a loss of behavioural thermoregulation. The reflex vasodilatation to heat is also impaired, though in response to extensive thermal stimulation some increase in tail temperature was noted [10].

The aim of the present experiments was to determine to what extent vasodilatation could be activated in capsaicin-treated thus desensitized [8] animals. Considering the close correlation between tail skin temperature and local blood flow [9] the temperature of the tail skin was regarded as an indicator of vasodilatatory response to heat.

Methods

Twenty-two control and 22 desensitized male CFY rats were used. Desensitization was performed at the age of 1 month with a total dose of 300 mg/kg capsaicin. The drug was administered subcutaneously in fractions of 10, 20, 20, 50, 100 and 100 mg/kg daily. The experiments started two months after this treatment. The rats were confined in a wire mesh restrain cage inside a test chamber of 24–26 °C. Radiant heat was used to warm the body: an infrared bulb of 62.5 W was placed at a distance of 30 cm on each side of the rat. The tail was shielded from the bulbs. Colon temperature (T_c) was taken by a platinum resistance probe inserted 6.5 cm into the colon. A disk thermistor was taped to the dorsal surface of the tail at two-thirds of its length to record tail skin temperature (T_t). Both T_c and T_t were taken at 1-min intervals.

After an adaptation period of 60–90 min, baseline temperatures were recorded for 15 min. The rats were then warmed to either 38 °C, 39 °C or 40 °C, and T_c was held at this level for 45 min. Heating was controlled by the colonic thermometer: by means of a regulating circuit, a deviation of ± 0.1 °C from the preselected T_c value switched the bulbs on or off. The sequence of the heat challenges was 39 °C, 38 °C and 40 °C for each rat, at intervals of at least 1 month. Mean $\pm$SEM body weight of the animals at the time of the tests was 39 °C: 449 $\pm$ 13

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g (controls) and 446 ± 12 g (desensitized); 38 °C: 465 ± 9 g (controls) and 464 ± 11 g (desensitized); 40 °C: 473 ± 10 g (controls) and 472 ± 12 g (desensitized). In the 38 °C test only those animals were used whose initial T_c was lower than 38 °C; thus, from the 22-member groups only 15 control and 19 desensitized rats were involved.

Results

The mean T_c ± SEM for each rat and test in the minute prior to heating was 37.2 ± 0.2 °C for the controls and 36.5 ± 0.2 °C for the capsaicin-treated rats, with a significant difference between them (Student's *t*-test). No difference was found in the T_t value (Fig. 1): It amounted to 27.1 ± 0.3 °C in both groups. Neither did the two groups differ in the time required to elevate their temperature to the preselected T_c.

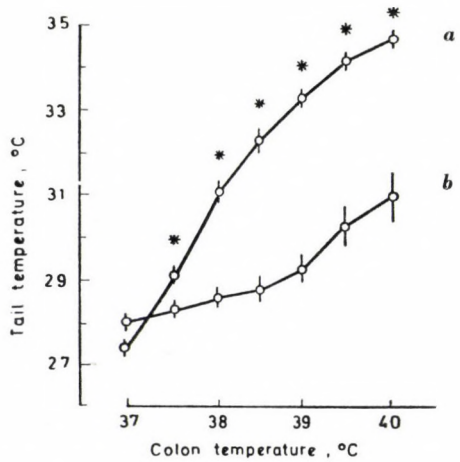

Fig. 1. Colon temperature and tail skin temperature during elevation of colonic temperature in control (*a*) and desensitized (*b*) rats. Asterisks denote significant differences of at least $p < 0.05$ (Student's *t*-test)

The relationship between body temperature and vasodilatation during the transient period of T_c elevation was characterized as described earlier [11]: The T_c values were grouped into classes spanning 0.5 °C intervals, and the mean T_t for each rat and for the three tests was calculated for each T_c class (Fig. 1). At a T_c of 37 °C no difference in T_t was observed between the control and capsaicin-treated animals. With continued heating T_t increased together with T_c in the control animals. In the desensitized rats, however, practically no vasodilatation was noted up to a T_c of 39 °C. Above this temperature the increase in T_t was obvious; the difference between the two groups was highly significant throughout the period of body temperature elevation.

While T_c was maintained at a hyperthermic level, the course of T_t depended on the extent of hyperthermia (Fig. 2). In the 38 °C test, in the control rats vasodilatation reached the maximum 10—12 minutes after the T_c of 38 °C had been established. When T_c was maintained at either 39 °C or 40 °C,

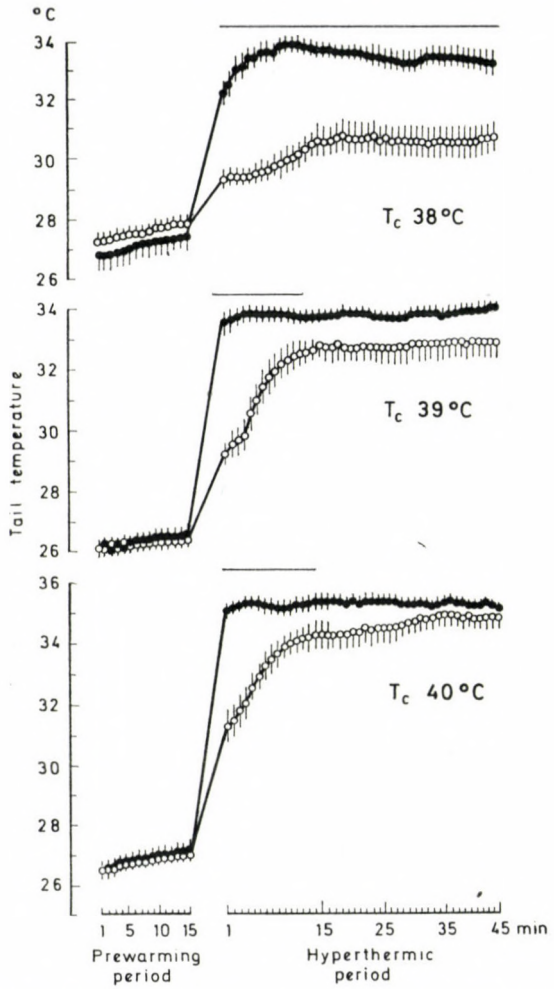

Fig. 2. Tail skin temperature in control (black circles) and desensitized (open circles) rats during the prewarming period and with body temperatures of 38 °C, 39 °C and 40 °C. Continuous lines above each pair of curves indicate the period during which tail temperature differed significantly in the two groups (Student's *t*-test, at least $p < 0.05$)

vasodilatation was complete during the transient period of body temperature elevation. The maximum increase in T_t developed in the desensitized rats long after the steady hyperthermia had been established. An important difference was, however, observed as regards the effects of the three heat challenges:

in the 38 °C test the vasodilatation was slight and remained significantly weaker than that of the controls throughout the experiment, while in the 39 °C and 40 °C tests T_t in the desensitized rats eventually reached the same level as in the controls.

Discussion

The findings supported the earlier observation of an impaired vasodilatation in capsaicin-desensitized rats. Vasodilatation was not simply impaired, but a definite T_c threshold could be determined: under 39 °C the response of the capsaicin-treated rats was significantly weaker than that of the controls, while above 39 °C the desensitized rats behaved like the controls, only the time necessary for development of the maximum reaction was different. The significance of this late vasodilatation in preventing severe hyperthermia is unclear. The heat challenge was excessive: both central and peripheral thermoreceptors were stimulated for a long time, while thermoregulation was not allowed to be effective. Still the existence of such elevated thresholds may contribute to an understanding of some earlier observations. Previously, in desensitized rats a general impairment of all thermolytic reactions was suggested. The thresholds for activating different thermoregulatory mechanisms by heat are, however, different even in the normal animal [4]. Accordingly, after capsaicin treatment, certain responses elicited at a high threshold in the normal rat are abolished, while responses of originally lower threshold can be — though at a higher T_c — activated. This may be one of the reasons for the controversial results found in studies of the various forms of behavioural thermoregulation in capsaicin-treated rats [2, 3, 6, 10, 11, 12], and this may explain why the desensitized rats demonstrated an increased sleeping time in a warm environment whereas the controls were active in grooming and trying to escape [1].

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EFFECT OF PROGESTERONE, TESTOSTERONE AND OESTROGENS ON THE PLASMA CORTICOSTERONE AND THYROID HORMONE CONCENTRATIONS IN THE FEMALE JAPANESE QUAIL

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Plasma concentration of thyroxine, triidothyronine and corticosterone has been determined by radioimmunoassay in short-day (6L—18 D) female Japanese quails. In the first experimental group Silastic capsules containing progesterone, testosterone, oestrone and 17-β-oestradiol, alone or in combination were implanted subcutaneously into ovariectomized birds. As compared to the ovariectomized group, plasma thyroxine concentration slightly increased following progesterone administration, while it decreased after testosterone, oestrone and 17-β-oestradiol treatment.

Both thyroxine and triiodothyronine levels showed a decrease after the simultaneous application of the four sexual steroids. Plasma corticosterone concentration was considerably increased by progesterone and 17-β-oestradiol administration, while oestrone caused an increase of smaller degree.

In the second experimental group, the same hormonal treatments were performed following ovariectomy and simultaneous surgical thyroidectomy. Plasma corticosterone concentration was found to be increased by the separate or simultaneous application of the sexual steroids, to the level observed in the intact control animals. According to our findings, plasma corticosterone conentration in the Japanese quail is increased by progesterone both directly and via the enhancement of the plasma thyroxine concentration, while it is directly increased by oestrone and oestradiol.

Earlier it has been shown [7] that corticosterone concentration of the blood plasma decreased following ovariectomy. Thus, an adrenocortical stimulatory effect of the ovarian sexual steroids had to be supposed to occur in female birds. This was in contrast with the finding in male Japanese quails where the corticosterone level was decreased by testosterone and increased by castration [5], an observation supported by histological data [1, 2, 4, 13].

The aim of the present investigation was to determine whether the plasma corticosterone level was increased by each of the ovarian sexual steroids or only by their combination and to observe whether their effect was directly realized at the adrenocortical level or indirectly by increasing thyroid secretion.

Material and Methods

Four weeks old female Japanese quails were kept in a short-day (6L—18D, start of illumination at 09:00) experimental room for 2 weeks. The birds were fed with fowl feed and water was allowed *ad libitum*. The intensity of illumination was 70 Lux/m^2.

At the age of 6 weeks, ovariectomy or ovariectomy + thyroidectomy were performed. After the intervention, progesterone, testosterone, oestrone and 17-β-oestradiol alone were implanted under the skin, and in a further experimental group, the four hormones were im-

planted simultaneously. The quantity of the implanted hormone(s) was regulated by the length of the implanted capsule, i.e. progesterone — 18 mm, testosterone — 6 mm, oesterone — 6 mm, 17-β-oestradiol — 8 mm.

On the 14th day the birds were bled by decapitation between 08:00 and 09:00 hours. Blood was collected into heparinized plastic centrifuge tubes and immediately centrifuged at 3000 rpm. The plasma was stored in polyethylene tubes at −20 °C until use.

The effect of the operation was checked at dissection. Only the plasma of birds in which no indication of a histological regeneration could be found was used.

Following extraction of the plasma by diethylether (1 : 10), the fractions were separated by Sephadex LH-20 chromatography [11], and progesterone, testosterone, oestrone and 17-β-oestradiol were determined by RIA [6]. Triiodothyronine and thyroxine were determined by direct RIA [9, 10]. To estimate corticosterone, the plasma was extracted by dichlormethane, then its corticosterone content was determined by RIA without previous chromatography. The binding capacity of the antibody diluted to 1 : 2000 was 35%; its cross-reactions were, cortisol, $<1.9\%$; progesterone, $<1.8\%$; cortexolene, $<1.1\%$; oestrone, $<0.004\%$; dexamethasone, $<0.002\%$.

For evaluation of the results, variance analysis was applied.

Result

In the *first series of experiments*, in the ovariectomized Silastic implanted birds, sexual steroid levels near to those characteristic of adult long-day quails were found (Table I).

Thyroid hormones. In sexually inactive Japanese quails, the plasma thyroxine concentration failed to change following ovariectomy, while the triiodothyronine level increased significantly. In ovariectomized birds, the thyroxine level increased after progesterone administration, while it showed a significant decrease after testosterone, oestrone and, first of all, 17-β-oestradiol treatment.

All these hormonal interventions failed, however, to affect the plasma triiodothyronine concentration. Compared with ovariectomized control birds, the thyroxine and triiodothyronine levels were significantly decreased by the simultaneous administration of the four sexual steroids. The plasma triiodothyronine level returned to the control value (Table II).

Corticosterone. Ovariectomy caused a significant decrease in the plasma corticosterone content. Progesterone and 17-β-oestradiol strongly, while oestrone slightly increased the corticosterone level in the ovariectomized quails. Testosterone implants had no effect on the corticosterone level, which increased considerably after the simultaneous application of all the sexual steroids. Their effect, however, was not additive (Table III).

In the *second series of experiments*, the effect of the individual sexual steroids was investigated in ovariectomized and thyroidectomized quails. In the case of implants applied one by one, the steroid concentrations were higher than in ovariectomized birds treated with steroids. On combined administration of the four steroids, however, the individual hormone levels were practically similar (Table IV).

Corticosterone. Following simultaneous ovariectomy and thyroidectomy the plasma corticosterone concentration decreased significantly while it was significantly enhanced by progesterone, testosterone, oestrone and 17-β-

Table I

Effect of sexual steroid implants on the sexual steroid levels in ovariectomized quails

pg/ml	18L (control)	6L (control)	OVx	OVx + PROG	OVx + TEST	OVx + E_1	OVx + E_2	OVx + 4 sex. ster.
PROG								
$\bar{x}$	1402	409	300	1547	—	—	—	1300
±SD	21	34	49	222	—	—	—	272
n	8	8	6	6	—	—	—	8
TEST								
$\bar{x}$	360	70	52	—	802	—	—	490
±SD	79	11	14	—	419	—	—	112
n	8	8	6	—	6	—	—	8
E_1								
$\bar{x}$	230	112	62	—	—	404	—	396
±SD	47	38	8	—	—	170	—	137
n	8	8	6	—	—	7	—	8
E_2								
$\bar{x}$	645	83	73	—	—	—	605	563
±SD	120	19	19	—	—	—	116	211
n	8	8	6	—	—	—	6	8

OVx, ovariectomy; OVx + PROG, ovariectomy + progesterone implantation in 18 mm Silastic capsule; OVx + TEST, ovariectomy + testosterone implantation in 6 mm capsule; OVx + E_1, ovariectomy + oesterone implantation in 6 mm capsule; OVx + E_2, ovariectomized + 17-β-oestradiol implantation in 8 mm capsule; OVx + 4 sex. ster., ovariectomy + PROG, TEST, E_1, E_2 in 4 Silastic capsules of size as given above; 18L, 18 h illumination; 6L, 6 h illumination.

oestradiol treatment. After their simultaneous administration, the plasma corticosterone level reached that of the control birds (Table V).

Discussion

In ovariectomized quails the plasma corticosterone concentration was considerably increased by the high sexual steroid level produced by hormone implants and characteristic of adult birds. This observation is in agreement with our earlier data, according to which the plasma corticosterone content decreased following removal of the ovary [7]. Progesterone and 17-β-oestradiol could be

Table II

Effect of sexual steroid implants on the plasma thyroid hormone content in ovariectomized quails

ng/ml	6L control	OVx	OVx + PROG	OVx + TEST	OVx + E_1	OVx + E_2	OVx + 4 sex. ster.
T_3							
$\bar{x}$	0.91	2.22[a]	2.92	2.40	2.84	2.74	0.87[b]
$\pm$SD	0.31	0.84	0.74	0.83	1.75	0.76	0.41
n	12	11	9	12	6	6	8
T_4							
$\bar{x}$	13.28	11.45	15.75[c]	8.14[c]	8.18[b]	3.01[b]	8.13[c]
$\pm$SD	2.82	3.32	1.36	3.97	2.11	3.01	3.03
n	12	11	9	12	6	6	8

Abbrevations as in Table I. [a] $p < 0.01$ vs 6L control, [b] $p < 0.01$ vs OVx, [c] $p < 0.05$ vs OVx.

responsible together for the effect of ovarian sexual steroids exerted on the plasma corticosterone level. Our findings are in agreement with those of RAMELEY [12] who found a decreased plasma corticosterone level in ovariectomized rats, while the level increased when 17-β-oestradiol was given following ovariectomy. Taking into consideration the effect of sexual steroids on the corticosterone secretion rate [8], corticosterone secretion seems to be stimulated by progesterone and 17-β-oestradiol first of all through the hypothalamo-corticotropic system, and the plasma glycocorticoid concentration is enhanced by the two sexual steroids. Plasma thyroxine concentration is influenced also by individual sexual steroids: it increased after progesterone and oestrogen administration. Plasma thyroxine concentration was found to be decreased if

Table III

Effect of sexual steroid implants on the plasma corticosterone in ovariectomized quails

ng/ml	6L control	OVx	OVx + PROG	OVx + TEST	OVx + E_1	OVx + E_2	OVx + 4 sex. ster.
CRT							
x	8.61	4.35[a]	10.89[b]	6.75	8.28[c]	14.80[b]	12.60[b]
$\pm$SD	2.16	2.27	4.77	4.23	1.32	5.55	2.37
n	8	6	8	7	7	6	8

Abbrevations as in Table [a] $p < 0.01$ vs control, [b] $p < 0.01$ vs OVx, [c] $p < 0.05$ vs OVx.

Table IV

Effect of sexual steroid implants on the sexual steroid concentration in ovariectomized and thyroidectomized quails

pg/ml	18L control	6L control	OVx + Tx	OVx + Tx + PROG	OVx + Tx + TEST	OVx + Tx + E_1	OVx + Tx + E_2	OVx + Tx + 4 sex. ster.
PROG								
x	1402	409	260	2137	—	—	—	979
±SD	21	34	27	320	—	—	—	310
n	8	8	8	6	—	—	—	8
TEST								
x	360	70	47	—	1221	—	—	690
±SD	79	11	10	--	210	—	—	273
n	8	8	8	—	9	—	—	8
E_1								
x	230	112	68	—	—	1370	—	750
±SD	47	38	23	—	—	205	—	291
n	8	8	8	—	—	6	—	8
E_2								
x	645	83	70	—	—	—	895	710
±SD	120	19	27	—	—	—	178	244
n	8	8	8	—	—	—	6	8

Abbreviations as in Table I. Tx, thyroidectomy.

Table V

Effect of sexual steroid implants on the plasma corticosterone (CRT) content in ovariectomized and thyroidectomized quails

ng/ml	6L control	OVx + Tx	OVx + Tx + PROG	OVx + Tx + TEST	OVx + Tx + E_1	OVx + Tx + E_2	OVx + Tx + 4 sex. ster.
CRT							
x	9.83	4.76[a]	8.57[b]	9.67[b]	9.13[b]	7.43[b]	8.61[b]
±SD	3.89	1.15	2.89	2.31	2.26	1.91	2.01
n	7	8	6	9	6	6	6

Abbrevations as in Table IV. [a] $p < 0.01$ vs control, [b] $p < 0.01$ vs OVx + Tx.

simultaneously the level of ovarian sexual steroids increased [6]. Since the plasma corticosterone concentration increased when thyroxine was given to sexually inactive female birds [PÉCZELY and PETHES, 7], there is a possibility that the corticosterone level was influenced by progesterone through a stimulation of thyroxine secretion. In mammals, where glycocorticoid production is enhanced first of all by 17-β-oestradiol, the effect of oestrogen on adrenocortical function depends on the actual functional state of the thyroid gland [3].

In ovariectomized and thyroidectomized animals the ovarian sexual steroids both individually and in combination were found to increase the plasma corticosterone level. This finding can be interpreted to indicate that in intact animals the plasma corticosterone concentration was increased by progesterone both directly through the hypothalamocorticotropic system and also by increasing the thyroid function. In the case of 17-β-oestradiol, the stimulation is direct in character, manifesting itself in spite of the decrease of the thyroxine level.

In ovariectomized birds, testosterone administration failed to influence the plasma concentration, while in simultaneously ovariectomized and thyroidectomized quails it caused a considerable increase in the corticosterone level. As to the effect of testosterone on adrenocortical function, the presence or lack of thyroid hormones was found to be of decisive importance also in male birds [5]. In hypothyroid quails, the plasma corticosterone level is increased by testosterone in both male and female birds.

Thus, it can be stated that in female quails both the plasma triiodothyronine and thyroxine concentration decreased following the simultaneous administration of ovarian sexual steroids in a dose maintaining the physiological state. This effect develops as a result of the slightly stimulating effect of progesterone and of the inhibitory effect of testosterone, oestrone and 17-β-oestradiol. The basis of the positive ovarian-adrecocortical connection is partly the direct effect of progesterone, oestrone and 17-β-oestradiol and partly the indirect effect of progesterone, realized through the stimulation of thyroid function.

Acknowledgement

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EFFECT OF INDOMETHACIN ON INTRARENAL CIRCULATION AND SODIUM AND WATER EXCRETION IN ANAESTHETISED RATS WITH OR WITHOUT ACUTE VOLUME EXPANSION

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Effects of the prostaglandin synthesis inhibitor indomethacin (4 mg/kg) were studied on the intrarenal circulation of anaesthetised (pentothal 50 mg/kg i.p.) rats in normovolemia or subjected to acute extracellular volume expansion (intravenous infusion of 0.9% NaCl at a dose of 50 ml/kg) using Sapirstein's ^{86}Rb indicator dilution technique. Circulatory parameters were determined one hour after indomethacin treatment. The following conclusions were drawn:

1. In normovolemic rats the renal, cortical and medullary blood flow remained unaltered. No changes occurred in regional vascular resistances of the kidney or in the distribution pattern of intrarenal blood flow. Water output was moderately reduced while sodium excretion remained unaffected.

2. In rats subjected to acute extracellular volume expansion renal cortical blood flow was slightly augmented, medullary perfusion rate declined; the intrarenal blood flow distribution was shifted towards the cortex. The vascular resistance in the cortex did not change whereas that in the medulla was slightly augmented. Under these conditions indomethacin did not influence salt and water excretion.

Our results provide further evidence that unlike in anaesthetised dog, in the anaesthetised rat endogenous prostaglandins probably do not play a decisive role in the control of renal blood flow, intrarenal circulation and salt and water excretion.

Prostaglandins (PGs) have been suggested to be involved as local hormones in the control of renal blood flow and in the regulation of renal salt and water excretion [3, 7, 12, 15, 17, 18]. Most of the conclusions concerning the role of prostaglandins have been drawn from experiments using indomethacin as the specific inhibitor of PG synthesis. The reduction of renal blood flow, the antidiuretic and antinatriuretic effects of indomethacin in the anaesthetised dog are well known [3, 7, 14, 16, 18]. In such dogs subjected to isosmotic extracellular hypervolemia indomethacin suppresses the diuretic and natriuretic effect of volume expansion [24]. However, in the anaesthetised rabbit indomethacin exerts differential effects, viz. it produces antidiuresis in hydropenia, whereas it markedly enhances the diuretic action of extracellular volume expansion [16, 19].

It appears that during anaesthesia the inhibition of endogenous PG production results in renal responses varying according to species and the state of hydration.

The purpose of the present study was to further elucidate the above phenomenon. The effects of indomethacin on renal function were studied in the anaesthetised rat with or without acute extracellular volume expansion.

Material and Methods

Female albino rats (CFY) weighing 200–240 g were used. Normal synthetic food and water were not restricted prior to the studies. Under pentothal anaesthesia (50 mg/kg i.p.) indomethacin (4 mg/kg) was injected via one of the tail veins. The indomethacin was dissolved in physiologic saline containing Na_2HPO_4 (1 mg indomethacin + 1 mg Na_2HPO_4/1 ml 0.9% NaCl). Thereafter the necessary surgical procedures were undertaken. Through a lower abdominal incision a catheter was inserted into the urinary bladder. A catheter was advanced into the right ventricle via the right jugular vein. The left common carotid artery was cannulated and connected to a T-tubing for monitoring the mean arterial blood pressure and collecting arterial blood samples at the final period of studies.

Surgery lasted for about 15 min. Cardiac output and parameters for renal haemodynamics were determined 60 min after the injection of indomethacin or solvent (control).

The studies were performed in four groups.

1. *Studies on normovolemic rats*

No infusion was given to the animals in these groups

1.1. Control group (n = 17)

1.2. Indomethacin group (n = 17)

2. *Studies on hypervolemic rats*

Forty min prior to the commencement of the circulatory study animals were infused with prewarmed physiologic saline (50 ml/kg) into the external jugular vein for 10 min. After completion of the infusion urine had been collected for 2×15 min periods and cardiac output and parameters for renal circulation were subsequently estimated.

2.1. Control group (n = 15)

2.2. Indomethacin group (n = 12)

3. *Estimation of circulatory parameters*

The animals received $^{86}RbCO_3$ (0.6—0.7 MBq) in 0.2 ml physiologic saline as a single bolus injected into the right ventricle via the jugular catheter. Arterial blood samples were collected at intervals of 0.75 s for 15 s. Animals were killed with i.v. aether 90 s after the isotope treatment. Kidneys were removed, decapsulated and specimens were excised from the cortex and medulla. These and the rest of the kidneys were weighed and dissolved in concentrated HNO_3. Radioactivity in blood and tissue specimens were determined in a gamma scintillation counter (Beckman Radioimmuno-Analyser). Cardiac output was determined on the basis of STEWART's principle [22], regional renal blood flow by SAPIRSTEIN's method [20] adapted for the kidney by HÁRSING and PELLEY [11].

Cardiac output (CO), renal blood flow (RBF), cortical blood flow (CBF) and medullary blood flow (MBF) were calculated according to the formula

$$BF = \frac{60 \times Q}{\int_0^t Ca\, dt}$$

where Q is the activity injected (for calculation of cardiac output, CO), and the activity calculated for 100 g of kidney, cortex and medulla (for calculation of RBF, CBF and MBF), respectively. The denominator $\int_0^t Ca\,dt$ represented the area below the arterial dilution curve the during first circulation.

The percentage distribution of intrarenal circulation was calculated from regional flow values per unit mass assuming that the cortex represented 64% and the medulla 36% of the total kidney mass. The kidney fraction of cardiac output was calculated as a quotient of tissue activity per injected activity.

Urinary sodium and potassium concentrations were determined by flame photometry (Digital Flame Photometer, OMSZÖV). Haematocrit was determined using a Hawskley microhaematocrit centrifuge.

Experimental data were analysed for significance by Student's paired *t* test.

Results

Since there was no preferential laterality in the circulatory parameters between the right and the left kidney in any of the studies, their averages were used. Table I presents means ±S.D. calculated from data of both kidneys.

1. *Effect of indomethacin in normovolemic (NV) rats*

In response to indomethacin treatment both mean arterial blood pressure ($p < 0.001$) and total peripheral resistance ($p < 0.01$) rose without any appreciable change in cardiac output. Total renal and regional blood flow and resistance were not affected by indomethacin. Fractional renal blood flow and the pattern of intrarenal blood flow distribution were also unchanged (Table I).

Urine flow ($V_{control}$: 1.52 ± 0.49; V_{indo}: 1.09 ± 0.28 µl/min/g, $p < 0.05$) was moderately reduced by indomethacin, whereas sodium excretion was not altered (Table II).

Table I

Effect of indomethacin on intrarenal circulation in normo- and hypervolemia

	Normovolemia		Hypervolemia	
	control n = 15	indomethacin n = 17	control n = 15	indomethacin n = 12
Blood pressure, mmHg	123 ± 9.9	144 ± 7.92***	125 ± 15.8	148 ± 14.2***
Cardiac output, ml/min/100 g	23.9 ± 5.9	22.6 ± 4.04	20.7 ± 4.13	22.2 ± 4.07
TPR, mmHg · ml^{-1} · s · kg^{-1}	32.2 ± 6.57	39.0 ± 6.83**	37.8 ± 8.84	41.1 ± 7.13
Renal blood flow, ml/min/100 g	367 ± 118	369 ± 84	494 ± 113^{++}	558 ± 89
Cortical blood flow, ml/min/100 g	402 ± 133	391 ± 69	554 ± 132^{++}	619 ± 96
Medullary blood flow, ml/min/100 g	233 ± 86	235 ± 53	281 ± 68	258 ± 61
Resistance				
R_{kidney} mmHg · ml^{-1} · s · kg^{-1}	2.18 ± 0.60	2.46 ± 0.55	1.63 ± 0.500^{+}	1.65 ± 0.358
R_{cortex} mmHg · ml^{-1} · s · kg^{-1}	2.00 ± 0.56	2.28 ± 0.35	1.46 ± 0.455^{++}	1.48 ± 0.311
$R_{medulla}$ mmHg · ml^{-1} · s · kg^{-1}	3.64 ± 1.43	3.89 ± 1.01	2.86 ± 0.813	3.68 ± 1.06*
Kidney fraction, %	11.8 ± 2.10	13.3 ± 2.66	15.9 ± 3.00^{+++}	16.8 ± 2.38
Distribution of RBF				
Cortex, %	78.3 ± 3.20	78.2 ± 2.86	80.2 ± 2.25	83.1 ± 1.90**
Medulla, %	21.7 ± 3.15	21.8 ± 2.92	19.8 ± 2.26	16.9 ± 1.90**

* Significance as compared with the initial values.
$^{+}$ Significance between the normo- and hypervolemic control groups.
*, $^{+}$: $p < 0.05$ **, $^{++}$: $p < 0.01$ ***, $^{+++}$: $p < 0.001$.

Table II

Effect of indomethacin on water and sodium excretion in normo- and hypervolemia

	Normovolemia		Hypervolemia	
	control n = 15	indomethacin n = 12	control n = 13	indomethacin n = 11
Diuresis, μl/min/g kidney	1.52 ± 0.49	1.09 ± 0.28*	18.4 ± 8.64^{+++}	18.9 ± 1.65
U_{Na}, μmol/ml	105 ± 25.6	185 ± 43.8***	232 ± 28.8^{+++}	241 ± 14.1
$U_{Na}V$, μmol Na/min/g kidney	0.16 ± 0.06	0.20 ± 0.054	4.24 ± 2.03^{+++}	5.37 ± 4.11

* Significance as compared with initial values.
$^{+}$ Significance between the normo- and hypervolemic control groups.
$^{*}_{+}$: $p < 0.05$ $^{***}_{+++}$: $p < 0.001$.

2. *Effect of indomethacin in hypervolemic rats (HV)*

Acute volume expansion per se did not influence either arterial blood pressure or cardiac output recorded 30 min after the termination of the infusion period. However, it did considerably (35%) increase renal perfusion rate (RBF_{NV} 367 ± 118, RBF_{HV} 494 ± 113 ml/min/100 g, $p < 0.01$). Cortical blood flow was augmented by 38% compared with normovolemic controls (CBF_{NV}: 402 ± 133, CBF_{HV}: 554 ± 132 ml/min/100 g, $p < 0.01$), whereas medullary blood flow was enhanced only by 20% (NS). Owing to these changes the distri-

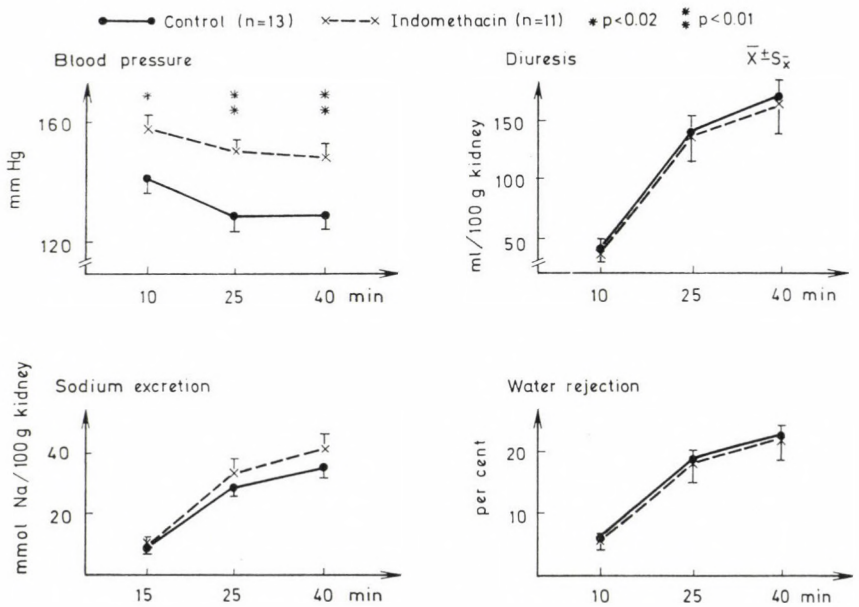

Fig. 1. Arterial blood pressure and salt and water excretion in rats with acute extracellular volume expansion under control conditions (●——●) and after the infusion of indomethacin (×– – –×)

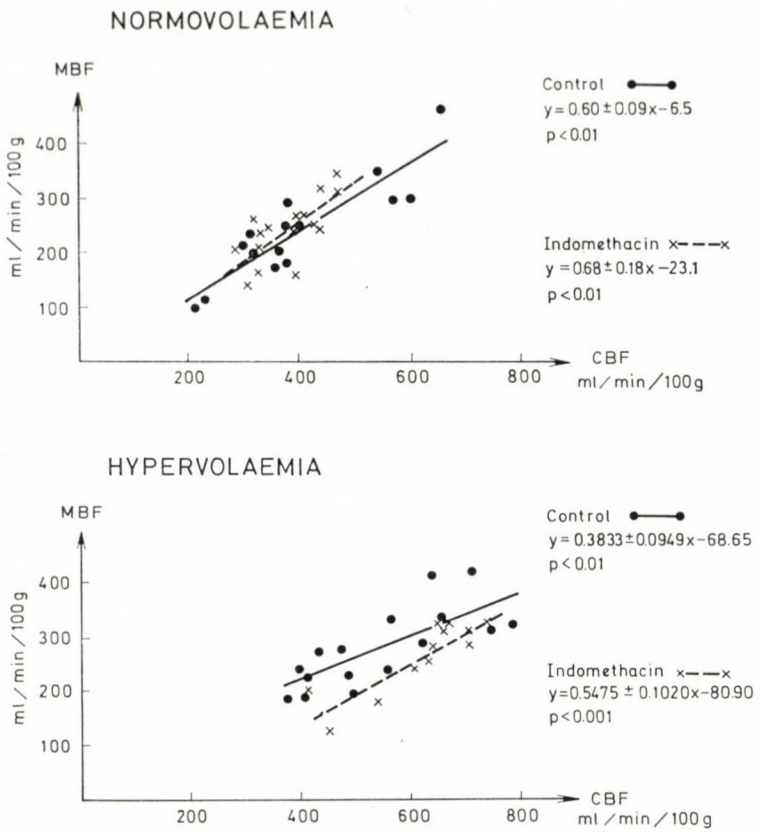

Fig. 2. Interrelationship between renal medullary and cortical blood flow in anaesthetised rats with normovolemia or hypervolemia under control conditions (●——●) and after the infusion of indomethacin (×– – –×)

bution of intrarenal blood flow tended to shift (NS) toward the cortex. Fractional renal blood flow was increased from 11.8 ± 2.1% to 15.9 ± 3.0% ($p < 0.001$) due to the acute volume expansion.

Indomethacin did not affect cardiac output in rats with acute volume expansion either, but it raised the arterial blood pressure ($p < 0.001$) just like in normovolemic rats (Table I and Fig. 1). Renal and renal cortical vascular resistance was not augmented by indomethacin but the drug did moderately increase the resistance of the medullary vessels ($R_{\text{medulla HV}} = 2.86 \pm 0.81$, $R_{\text{medulla HV + indo}} = 3.68 \pm 1.06$ R; $p < 0.05$). Cortical blood flow tended to increase, medullary blood flow tended to decrease but changes were not statistically significant. Percentage distribution of intrarenal blood flow shifted toward the cortex, i.e. it increased from 80.2 ± 2.25% to 83.1 ± 1.9% ($p < 0.01$), whereas the same parameters in the medulla decreased from 19.8 ± ± 2.26% to 16.9 ± 1.90% ($p < 0.01$) under the effect of indomethacin.

Figure 2 presents changes in intrarenal blood flow distribution, medullary blood flow being plotted against cortical blood flow. In normovolemic animals the regression lines for both parameters were almost indentical. In the volume-expanded rats the regression line for the indomethacin-treated rats was shifted downward indicating that after indomethacin treatment any given cortical flow value was associated with lower medullary flow value than in controls.

In rats with acute volume expansion indomethacin failed to influence urine flow, sodium output and fluid excretion as expressed in per cent of the volume of fluid administered (Fig. 1).

Discussion

In the anaesthetised dog the renal vasoconstrictor effect of indomethacin has repeatedly been confirmed. In the anaesthetised and operated normovolemic dog indomethacin treatment results in potent renal vasoconstriction [3, 7, 15, 17, 18, 23]. The indomethacin-induced renal vasoconstriction is also observed in anaesthetised dogs subjected to isosmotic extracellular volume expansion and at the same time the enhancement of salt and water excretion due to the hypervolemia is suppressed [24]. There is a linear relationship between the reduction of renal blood flow and the release of PGE_2 from the kidney in dogs [18, 23]. Therefore, it is suggested that the continuous basal PG secretion might play a role in the control of renal blood flow.

On the basis of these findings it appears that in the anaesthetised dog the onset of the indomethacin-induced renal vasoconstriction is independent of the state of hydration. The aim of the present investigation was to study the actions of indomethacin in rats with or without acute volume expansion.

In the anaesthetised normovolemic rat —in contrast to the dog under similar conditions— there was no change in renal and intrarenal circulation including medullary flow after indomethacin treatment. This finding is in good agreement with data of GANGULY et al. [9] who found no changes in the plasma flow of the papilla after indomethacin administiation, and also with those of DÜSING et al. [6] who observed unaltered GFR and renal plasma flow as measured by ^{135}I hippurane after indomethacin treatment in rats on a normal salt diet. On the other hand, our results are not in line with those of SOLEZ et al. [21] who reported on a marked reduction of medullary plasma flow measured by the ^{125}I-albumin method 30–45 min after intraperitoneally injected indomethacin. According to their results total renal blood flow was reestablished one hour following indomethacin treatment despite the inhibition of PG synthesis which persisted for one and a half hour posttreatment. According to SOLEZ et al. [21]

the effect of indomethacin administered into the renal arteries is relatively short-lived. We gave indomethacin intravenously and found no change in renal haemodynamics one hour postinjection. HUCKER et al. [13] found that the plasma half-life of indomethanic administered i.v. was 4 h, therefore the PG producing cells were supposed to be under the effect of indomethacin throughout the whole observation period of the present study. This presumption is supported by the fact that under normovolemic conditions both arterial blood pressure and total peripheral resistance were potently increased by indomethacin. Previous results from this laboratory have shown that the enhancement in TPR is caused by a preferential increase of vascular resistance in the skin and skeletal muscle [4].

On the basis of the present results and the above data we suggest that in anaesthetised normovolemic rats the rate of the basal prostaglandin secretion is low, therefore it should not play an important role in the control of renal and intrarenal blood flow. It appears, however, that the state of hydration and/or the extent of sodium load can modulate the responses of intrarenal haemodynamics to indomethacin treatment even in rats.

The diuretic and natriuretic effects of prostaglandins of the E and A series after their infusion into the renal arteries are well known [2, 10, 25]. If the endogenous prostaglandins played a role in the volume regulation as natriuretic factors, their secretion would be expected to enhance during acute isosmotic extracellular volume expansion. We tested the validity of this presumption in rats with *hypervolemia*. In hypervolemia rats following treatment with the prostaglandin synthesis inhibitor indomethacin cortical blood flow was moderately increased whereas medullary flow was slightly reduced despite the elevated blood pressure. These changes, however were not statistically significant. On the other hand, vascular resistance in the medulla significantly increased after indomethacin treatment (medullary blood flow slightly decreased in association with the elevation of blood pressure), however, its biological importance remains doubtful. Thus, indomethacin failed to induce marked renal vasoconstriction in anaesthetised rats even during hypervolemia, which is in contrast to results described for anaesthetised and operated dogs under similar conditions.

In the present study indomethacin in hypervolemic rats caused a shift in intrarenal blood flow distribution toward the cortex, just opposite to the changes observed in rats with normovolemia. This finding is similar to that found in anaesthetised dogs [3, 15]. The mechanisms of redistribution in the two species are, however, different. In the dog the cause of the outward redistribution after indomethacin treatment is that the vasoconstriction in the outer cortex is smaller in magnitude than in the juxtamedullary cortex or in the medulla [3, 15]. Based on our present results the cause of the redistribution in the rat is that the moderately reduced medullary perfusion is associated with a slightly enhanced cortical flow.

Results also showed that the endogenous prostaglandin secretion did not appear to play a decisive role in the control of cortical blood flow in anaesthetised rats either with or without acute volume expansion. The indomethacin-induced responses in the medullary vessels depended on the state of hydration. In normovolemia due to the presumably low endogenous PG secretion the further reduction of PGs did not cause the medullary flow to decline, in rats with acute volume expansion, however, the suppression of the enhanced prostaglandin production might have caused a slight increase in the resistance of medullary vessel inducing a moderate reduction of flow through this zone. Nevertheless, the characteristic changes in renal haemodynamics induced by indomethacin in the anaesthetised dog failed to occur under similar conditions in rats with or without acute volume expansion. Our results provide further evidence for the differential vascular effects of prostaglandins in the kidney of the two species.

The endogenous prostaglandins might modulate sodium and water excretion through the control of renal haemodynamics and/or of tubular functions. Indomethacin did not alter sodium output in rats either in normovolemia or hypervolemia. Urine flow in normovolemia was reduced by 29% after indomethacin treatment, however, no such effect was observed in rats subjected to volume expansion (Table II). These findings should not be ascribed to haemodynamic changes (Table I) but can be readily interpreted in terms of the opposing actions of ADH and PGE on the enzyme adenylate cyclase: vasopressin facilitates whereas PGE inhibits adenylate cyclase. The vasopressin potentiating effect of indomethacin is well known in the anaesthetised dog [1, 8]. PGE suppresses the vasopressin-induced increase of adenylate cyclase activity and of cAMP concentration in the medulla of the rat kidney [5]. In normovolemia we also found reduced water output and increased urinary sodium concentration (Table II). Therefore, the indomethacin-induced reduction in water output observed in normovolemic rats appears to be the result of a predominance of the effect of vasopressin owing to the suppression of endogenous prostaglandin production.

On the other hand, in hypervolemia urine flow was not diminished by indomethacin. This does not appear to be in conflict with our explanation based on the vasopressin-prostaglandin antagonism. Apart from plasma osmolality, hypervolemia is the most potent inhibitor of vasopressin synthesis. Data for urinary excretions in Table II are derived from the last 15-min collection period prior to the haemodynamic study. Thus by that time already 30 and 45 min had elapsed from the onset of hypervolemia and indomethacin injection, respectively. Accordingly, the secretion of vasopressin must have been suppressed to such a degree that with the plasma $T_{1/2} = 18$ min for the hormone, indomethacin (i.e. the lack of prostaglandins) could not potentiate the effect of vasopressin.

Our results provide further evidence that in the anaesthetised rat the endogenous prostaglandins probably do not play such a decisive role in the control of renal blood flow, interarenal circulation and in the salt and water excretion as in the anaesthetised dog. The renal actions of the prostaglandin synthesis inhibitor indomethacin thus vary according to species and the experimental conditions applied.

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EFFECT OF IMMOBILIZATION BY PLASTERING ON THE LEVEL OF VARIOUS ENERGY METABOLITES IN SKELETAL MUSCLE

By

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The effect of 1 to 6-week limb immobilization by plaster fixation and the resulting skeletal muscle inactivity-atrophy was studied by analysing isotachophoretically the ATP, ADP, AMP, CrP, NAD^+ and P_i content of the slow-oxidative soleus, and the fast-glycolytic gastrocnemius muscle of the rabbit.

ATP and CrP levels were higher in the glycolytic while NAD^+ content was higher in the oxidative skeletal muscle under normal conditions. Energy homeostasis of the muscles disturbed during the first week of immobilization, and subsequently it is adapted to the inactivity by reaching a new steady state at lower energy levels. This new level as characterized by the high energy phosphate content shows no significant difference between the two muscle types.

Experimental inhibition of skeletal muscle activity induces considerable ultrastructural and biochemical changes in this tissue. These changes are, however, reversible depending on the type of the muscle. Elimination of the activity-blocking factor leads to regeneration of the muscle. Limb immobilization by plaster fixation gives rise to typical inactivity atrophy in skeletal muscles if it is sustained for a sufficiently long period [1, 2]. Morphological and biochemical background of this process has been studied in detail [2–5, 7, 10–13] due to its medical and social rehabilitation importance.

Although the changes in the energy stores of mammalian skeletal muscle during various pathological conditions have been well defined [6, 8, 9], the alterations in energy metabolism following immobilization and during the consequent muscle atrophy have not been clarified yet.

Materials and Methods

Tissue samples and preparation

Muscle samples of 40 to 70 mg wet weight were excised from male New-Zealand rabbits (2—2.5 kg body weight) (n = 23), anaesthetized by Nembutal. The excised samples were placed immediately into liquid nitrogen, followed by extraction in 3 ml 50% methanol (pH = 7.6) containing 1.25 mM EDTA, at —30 °C for 96 hours. Wet weight of the samples was measured immediately after finishing extraction. Methanol was removed from the extract in vacuum at 0 °C. The solutions were thereafter transferred into precooled 5 ml Kimbl-vials, frozen at —30 °C and stored at —20 °C until further analysis. Liophylized extracts were dissolved in distilled

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water (3 ml) at 4 °C before isotachophoretic analysis, and bacteria and filaments were removed by a Sartorius (SM 11324) membrane filter.

Determination of ATP, ADP, AMP, CrP, P_i and NAD^+ content of muscle extracts by isotachophoresis

Ten microliter (μl) portions of samples stored and treated as described above were analysed by an LKB capillary analytical isotachophoretic equipment (LKB Tachophor 2127).

The following criteria were met during analysis: length of the capillary, 230 mm; inner diameter, 0.5 mm; leading electrolyte, 5 mM Cl^- (Suprapure, Merck) + beta-alanin (Serva) (pH: 3.99) + 0.4% HPMC (Dow Chem. Co.) (hydroxypropylmethylcellulose); terminating electrolyte, 5 mM caproic acid (BDH); temperature, 20 °C; current intensity, 65 μA; voltage, 8—12 kV; recording, UV 254 nm; paper speed, 6 cm/min; analysis time, 12 min.

The values were expressed as arithmetic means ± SD. Statistical analysis of the data was expressed using Student's *t* test.

Results

The concentration of metabolites was compared in the two muscle types under control untreated conditions (Fig. 1). The differences between NAD^+, CrP and ATP content of the two muscle types in the normal state was statistically significant ($p < 0.05$). High-energy phosphate ester content (in the form of CrP and ATP) of the fast muscle (m. gastrocnemius) was higher by 70% than that of the slow muscle (m. soleus). In contrast, NAD^+ content was higher in the slow muscle.

Figures 2 and 3 illustrate the relationships between ATP, ADP, AMP, CrP, P_i and NAD^+ content and the time of immobilization in soleus muscle (oxidative type). Peak changes of ATP, AMP, P_i and NAD^+ content of the slow muscle occurred in the first week of immobilization.

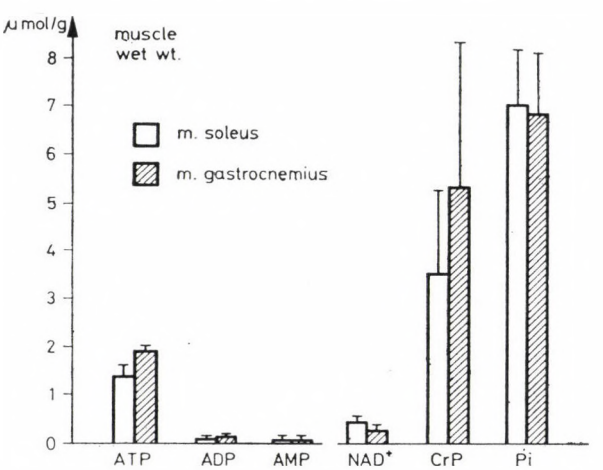

Fig. 1. ATP, ADP, AMP, CrP, P_i and NAD^+ content of methanol extracts of soleus and gastrocnemius muscle under normal conditions. Residual high-energy phosphate content determined enzymatically [15] was as follows: ATP, 50%, ADP and CrP, 30%

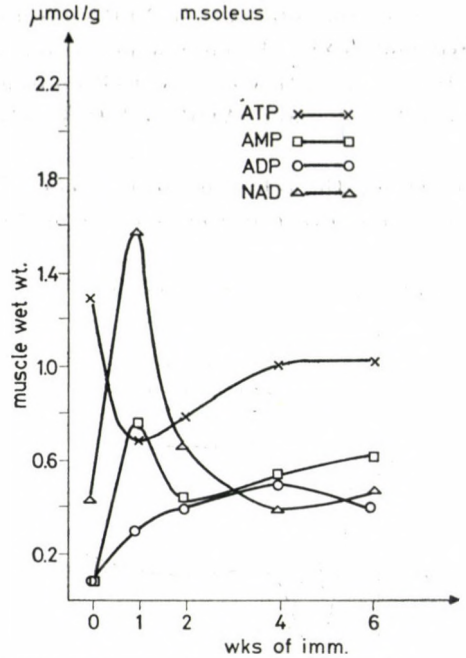

Fig. 2. Changes of ATP, ADP, AMP and NAD^+ levels in the soleus muscle as the function of immobilization time

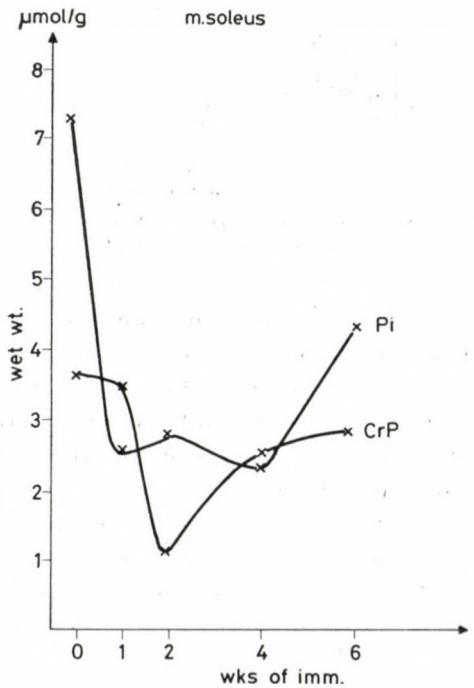

Fig. 3. Changes of CrP and P_i content of the soleus muscle during immobilization

In the first week of immobilization ATP content reached a minimum (48 $\Delta\%$), while AMP and NAD^+ levels reached a maximum (1010 $\Delta\%$ and 270 $\Delta\%$, respectively) (Fig. 2). In contrast, CrP content reached a minimum (66 $\Delta\%$) on the second week, and ADP and P_i levels on the fourth week (68 $\Delta\%$) (Fig. 3).

The curves describing the relationship between ATP, AMP, CrP and P_i levels and the time of immobilization were similar in the case of the glycolytic

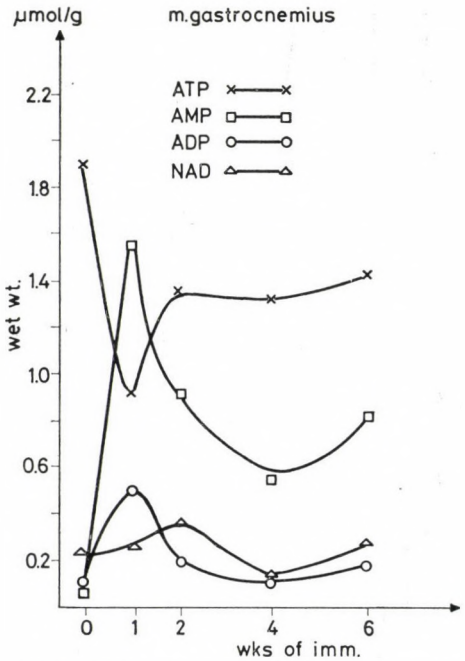

Fig. 4. Changes of ATP, ADP, AMP and NAD^+ levels in the gastrocnemius muscle as the function of immobilization time

muscle (Figs 4 and 5). At the same time the change of ADP content reached its maximum on the first week and NAD^+ content remained unaltered during immobilization. The changes of the individual parameters (with the exception of CrP, the change of which was not significant) were statistically significant only in the first week of immobilization in both muscles.

Figure 6 demonstrates the variations in the total adenine nucleotide phosphate content as the function of immobilization time in the two muscle types. Total nucleotide phosphate level increased gradually during immobilization in the soleus muscle and it stabilized in the 4th to 6th week. In the glycolytic gastrocnemius muscle the nucleotide phosphate level decreased gradually after the initial rapid increase observed in the first week and returned to control level in the fourth week of immobilization.

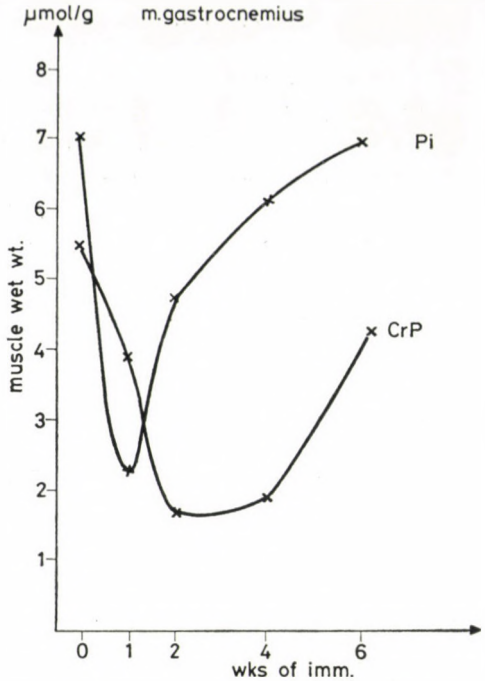

Fig. 5. Changes of CrP and P_i content of the gastrocnemius muscle during immobilization. The experimental results demonstrated in Figs 2–5 were not statistically significant

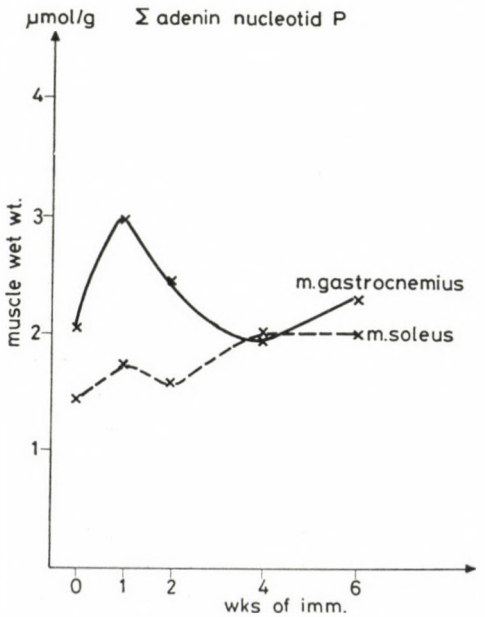

Fig. 6. The change of total adenine nucleotide phosphate content in the soleus and gastrocnemius muscles during immobilization

Discussion

So far no data have been available about the energetic sequelae of immobilization and consequent atrophy in mammalian muscles.

According to the present results immobilization induces considerable changes in both the fast and slow skeletal muscles. The significant decrease of ATP, P_i and CrP content in the first and second week of immobilization was striking. The changes may have been the result of several mechanisms, the most important of which may be the washout of inorganic phosphate from the tissue, accumulating and released from the cytoplasm due to ATP and CrP

Table I

Effect of immobilization on the ATP, ADP, AMP, CrP, P_i and NAD^+ content of the soleus and gastrocnemius muscles (the values are expressed in μmol/g muscle wet weight)

	ATP	ADP	AMP	NAD+	CrP	P_i
M. soleus						
normal	1.29±0.19	0.061±0.011	0.068±0.014	0.42±0.097	3.6±1.9	7.25±1.14
1 week	0.68	0.302	0.755	1.56	3.37	2.51
2 weeks	0.78±0.41	0.39±0.198	0.41±0.202	0.66±0.47	1.23±1.01	2.76±1.02
4 weeks	1.00±0.43	0.5±0.29	0.53±0.27	0.39±0.21	2.42±1.73	2.36±1.21
6 weeks	1.04±0.28	0.37±0.06	0.61±0.12	0.47±0.17	2.68±1.65	4.27±2.605
M. gastrocnemius						
normal	1.9±0.15	0.074±0.034	0.082±0.030	0.225±0.053	5.47±3.4	7.09±1.23
1 week	0.93	0.49	1.55	0.26	4.1	2.28
2 weeks	1.35±0.40	0.19±0.06	0.9±1.21	0.37±0.23	1.71±0.16	4.7±1.67
4 weeks	1.31±0.16	0.13±0.09	0.55±0.33	0.14±0.08	1.87±0.08	6.12±2.21
6 weeks	1.425±0.101	0.17±0.11	0.82±0.27	0.27±0.07	4.23±1.603	6.98±0.20

breakdown as a consequence of reduced energy requirements in the immobilized muscle. This probably leads to deficiency in the amount of substrate for the synthesis of high energy phosphates in subsequent periods of immobilization.

The ratio of ATP/P_i remained constant during immobilization in both muscle types (Table II). The ratio of ATP/ADP + P_i, which is inversely related to the rate of adenine nucleotide phosphate synthesis, decreases gradually. The ratio of total adenine nucleotide phosphate pool and inorganic phosphate increases reaching its maximum (4-fold increase) during the first week of immobilization in the soleus muscle and remaining on this level until the 6th immobilization week. In the gastrocnemius muscle the increase during the first week (also a 4-fold elevation) is followed by a continuous drop reaching the

Table II

Changes of characteristic quotients of the energy supply in the skeletal muscles during different periods of immobilization

	CrP/ATP	ATP/ADP + AMP	ATP/P_i	Total adenine nucl.-P/P_i
M. soleus				
normal	2.79	10	0.17	0.195
1 week	4.95	0.64	0.27	0.69
2 weeks	1.57	0.975	0.28	0.57
4 weeks	2.42	0.97	0.42	0.86
6 weeks	2.57	1.06	0.24	0.47
M. gastrocnemius				
normal	2.87	12.12	0.26	0.29
1 week	2.45	0.45	0.40	1.3
2 weeks	1.26	1.23	0.28	0.52
4 weeks	1.42	1.92	0.21	0.32
6 weeks	2.96	1.44	0.20	0.34

control value in the fourth immobilization week. These changes indicate that there is an increased adenine mono- and diphosphate synthesis during immobilization in the soleus muscle.

Our data suggest that the muscle is able to adapt to changes in the physiological condition, i.e. to immobilization attaining a new steady state at a lower energy level. Although the concentration of high energy metabolites ATP and CrP does not reach the normal value the energy supply of the muscle improves considerably by the 4th to 6th week of immobilization, the mechanism of which is completely unexplained yet.

This adaptation, however, leads to successive abolition of the specific differences in the ATP, CrP, NAD^+ and total adenine nucleotide levels between slow-oxidative and fast-glycolytic skeletal muscles, since the difference between the two muscle types in this respect was significantly less pronounced after the 6th week of immobilization than under control conditions.

This "dedifferentiation" effect of immobilization in various types of skeletal muscle has already been described with respect to energy supplying enzymes [10], contractile properties [14] and structural proteins [11–13].

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NEW THEORY OF THE DEVELOPMENT OF MYOCARDIAL INFARCTION

By

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Solutions of KCl (1%, 10% and 31.2%) administered into the pericardial fluid or applied onto the surface of the heart evoked a dome-like change of the ECG in dogs, rats and guinea-pigs and led to myocardial infarction in 3—5 days. Both the acute changes in ECG and the infarction itself could be prevented by the application of pericardial fluid samples and by administration of three synthetic compounds onto the heart surface. The same substances also inhibited the development of ECG changes elicited by general hypoxia due to stopping artificial respiration. The existence of a peculiar myocardial space beginning with outer pores and reaching the myocardial cells through connective tissue pathways is postulated.

Earlier studies showed that ^{125}I-labelled albumin applied to the epicardial surface through a filter paper reached the endomyocardium through some intramyocardial pathways beginning with epicardial pores.

In the present experiments intrapericardial application of India ink led to obstruction of these pores and thus prevented the infarction elicited by intrapericardial administration of KCl solutions. This space, being distinct from that accessible from the coronary arteries, serves for transfer of various substances into the myocardium, while other compounds (e.g. noradrenaline) are not effective through this pathway. Oxygen reaching this space from the epicardial surface protects the myocardium from the damaging effects of hypoxia and KCl.

Ten years ago we reported on the existence of peculiar pores localized in the visceral pericardium [8]. Their detection had been based on the finding that almost 10% of the activity of ^{125}I-labelled albumin applied on the surface of the heart through a filter paper could be detected in the endocardium and endomyocardium. From this unexpected result it was concluded that the iodinated albumin had to reach the endocardium from the epicardium by some unknown pathways, since the size of the albumin molecule excluded the possibility of diffusion. Alternatively, as a possible route for the transport of labelled protein into the endocardium and its surroundings, the existence of preformed epicardial pores and some as yet unidentified connective tissue passages have been postulated, which could function as alternative channels to bypass the high coronary flow.

It seemed, therefore, of interest to study this non-coronary space and its possible role in pathological processes.

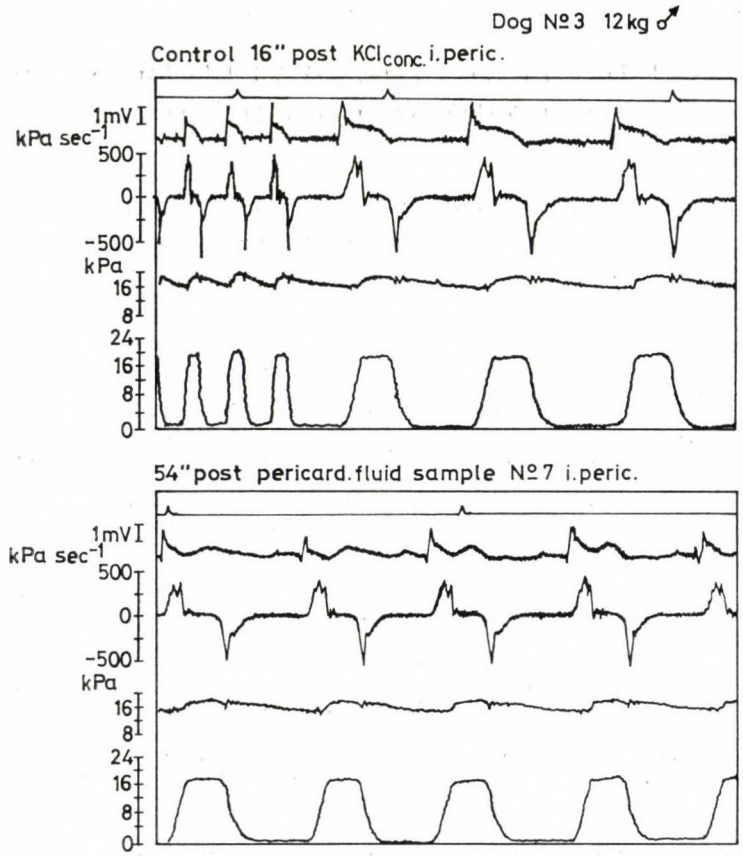


Fig. 1. Effects of KCl administered into the pericardial fluid (upper part). A 0.05 ml sample of pericardial fluid inhibits the ST-elevation 54 s after the injection of KCl (lower part). Abscissa: time in s. Ordinate (from above): ECG lead II, dP/dt, aortic pressure, left ventricular pressure

Methods

In the investigations 22 dogs, 234 rats and 44 guinea-pigs of both sexes were used.

Experiments in dogs. Solutions of KCl (1%, 10% and 31.2%) were injected into the pericardial sac. Electrocardiogram (lead II), blood pressure, left ventricular pressure, end-diastolic pressure and the first derivative of the left ventricular pressure were registered on an Elema—Schönander Mingograph Type 81. In some experiments the KCl solutions were applied through a window cut on the pericardium directly onto the epicardium by means of a piece of filter paper 18–22 mm in diameter previously soaked in the KCl solution. During the experiments pericardial fluid samples were taken and tested on the hearts of dogs, rats and guinea-pigs (for details see below).

It seemed to be important to study a possible role of oxygenation through the coronary pace. To study such a likelyhood a thin needle was passed into the pericardial sac to introduce pure oxygen from a flask, while a somewhat thicker needle was used to lead the oxygen out, ensuring thus a standard intrapericardial (i.peric.) pressure during steady inflow of 20–25 litre/h of oxygen, monitored by a rotameter.

In experiments studying the effect of epicardial application of KCl (filter paper soaked in KCl solution), oxygen was led directly onto the surface of the heart via a needle fitted with

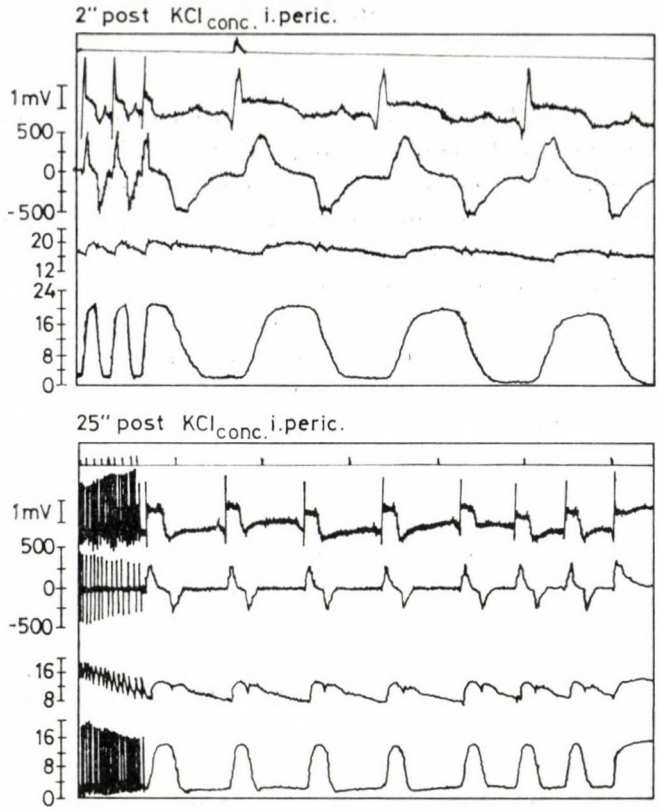


Fig. 2. The vagus-like effect of 31.2% KCl solution. Tracings as in Fig. 1. Note: in the majority of cases the changes in blood pressure were less marked

a multiply perforated nozzle 6 mm in diameter. The effect of epicardial oxygenation was studied during general hypoxia due to stopping artificial respiration or during clamping of the anterior descendent (LAD) or circumflex branch (LC) of the left coronary artery for 1 to 2 min.

Experiments in rats and guinea-pigs. In these species i.peric. administration of KCl and other solutions (0.05 ml) was carried out by thoracotomy or by piercing the injection needle through the diaphragm into the pericardial sac after opening the abdominal wall just below the xyphoid process. ECG (lead II) was recorded by an Elema—Schönander Mingograph Type 81. The effect of KCl and its blockade by pericardial fluid samples were investigated. Since the possibility of transfer of some protective substances both from the pericardial space to the myocardial cells and vice versa could be supposed, pieces of the parietal pericardium were cut out of dogs, rats and guinea-pigs and incubated in Krebs solution for 1 h to 1–2 days. These incubated solutions were then administered to the hearts of the respective species and the possible protection afforded against the influence of KCl was studied.

An attempt was made to obstruct the postulated pores by India ink, thereby preventing KCl to penetrate these pores.

In addition, some synthetic compounds (substances X, Y and Z*) were tested for their effect on the action of KCl. Furthermore, the time-course of infarction was investigated by staining myocardial sections with haematoxylin-eosin. For the detection of hypoxic spots, Vértesi's and Szentiványi's [9] special staining was applied. For statistical evaluation of paired and unpaired samples Student's *t*-tests were used.

* X, Y and Z denote synthetic substances. X: Histoacril blau (Braun Melsungen), Y and Z: methyl and aethyl esters of acrilic acid, respectively.

Results

Experiments in dogs

a. Fig. 1. shows that i. peric. administration of concentrated (31.2%) KCl solution evoked a dome-like change of ECG, characteristic of myocardial infarction. The change appeared some seconds after the injection and lasted for 20 to 35 min. Injection of more dilute solutions of KCl (1% and 10%) brought about a similar ECG effect of shorter duration. Local application of KCl to the surface of the heart led to similar changes indicating that such a local action of KCl might occur also in circumscript manifestations characteristic of human hypoxic heart disease. KCl applied either intrapericardially or locally was effective in the vast majority of cases in evoking a monophasic ECG change (in almost 100%).

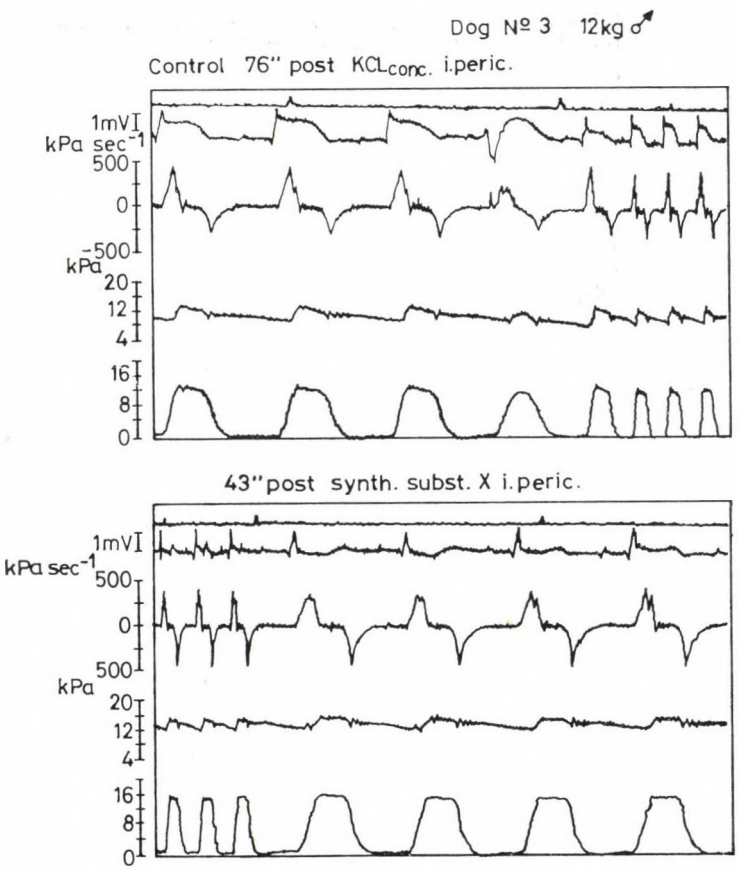


Fig. 3. Effect of a synthetic substance (Substance X) on the rise in ST-segment evoked by KCl. Upper part: i.peric. administration of $KCl_{conc.}$ Lower part: 43 s after the injection of substance X ST-segment has normalized and blood pressure rises. Tracings as in Fig. 1

b. As a next step, we were interested to know if the pericardial fluid contained any substance that could inhibit the effect of KCl. Fig. 1. demonstrates that pericardial fluid samples prevented the KCl-induced changes. As shown in Fig. 2, blood pressure could also be lowered. In most cases such changes were negligible.

c. The synthetic substances X, Y and Z provided similar protection as did the natural constituents of the pericardial fluid (Fig. 3).

d. Another series of experiments was aimed at studying whether a special space of myocardial fibres thought to be approached via the epicardial pores was responsible for the observed effects of KCl. An attempt was made to influence the effect of KCl by oxygen introduced through the pericardial space. Thus, there may exist a peculiar effect of oxygen characteristic of this space only. Figure 4 shows that the monophasic action potential evoked by KCl

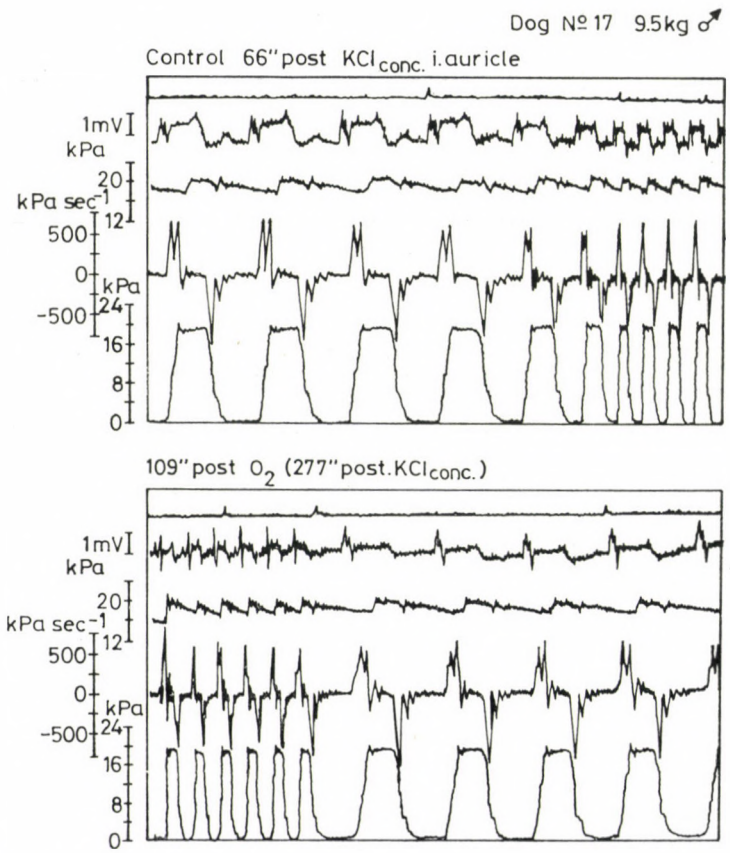


Fig. 4. Surface oxygenation of the epicardium. Upper part: ST-elevation after KCl (dome). Lower part: Reduced ST-elevation after oxygen administration for 109 s. Tracings as in Fig. 1. KCl and oxygen were applied to the left auricle

was normalized shortly after the pericardial introduction of oxygen. The rise in blood pressure induced by general hypoxia could also be prevented by administration of oxygen to the surface of the heart (Fig. 5). Figure 6 demonstrates that after administration of oxygen to the epicardial surface the increase in end-diastolic pressure evoked by hypoxia and KCl administration was reduced and after the termination of oxygen treatment the end-diastolic pressure rose again. Hence, oxygen is active in the above-mentioned space by counteracting the effect of hypoxia and KCl.

The same applies to local hypoxia induced by clamping one of the coronary arteries. As shown by Fig. 7, clamping of the coronary circulation caused a depression of the ST-segment, an effect opposite to that of i. peric. administration of KCl.

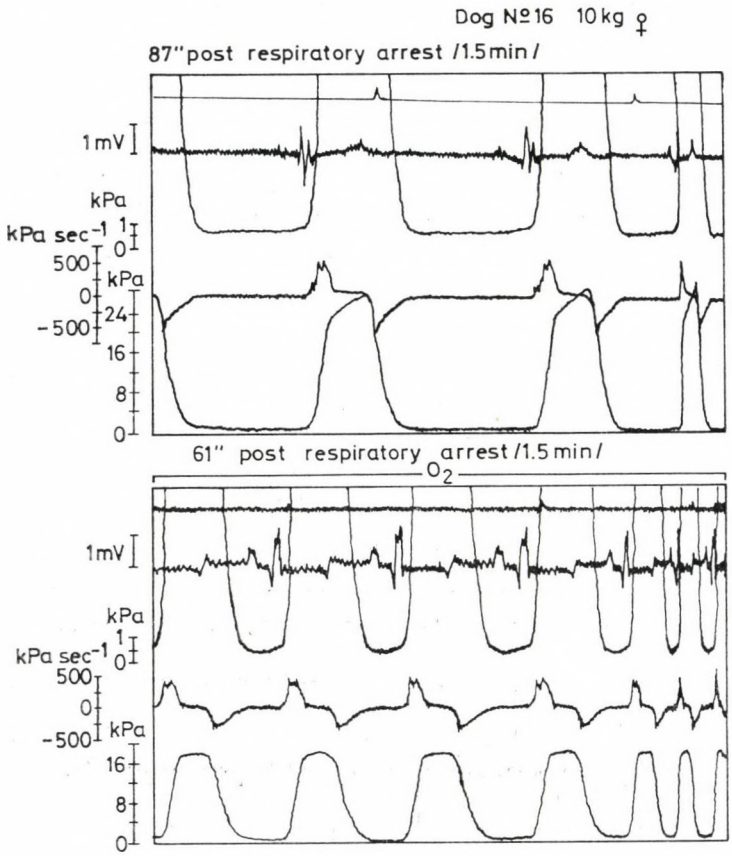


Fig. 5. Effect of oxygenation of the epicardial surface on the augmentation of the left ventricular pressure and end-diastolic pressure evoked by general hypoxia (asphyxia). Tracings (from above): ECG lead II, left ventricular end-diastolic pressure, dP/dt, left ventricular pressure. Time 1 s. Upper part: increased end-diastolic pressure and ventricular pressure after 1.5 min respiratory arrest. Lower part: during oxygenation of the heart surface no rise in end-diastolic pressure and ventricular pressure can be observed

These results are summarized in Tables I to III. It can be seen that the substances applied had practically no effect of their own on the circulation (Table I), while the biological and synthetic substances applied reduced the T/2 values of the KCl effect (Table II). The rise in blood pressure caused by asphyxia was diminished by surface oxygenation of the heart (Table III).

The finding that substances such as noradrenaline administered into the epicardial space failed to influence the circulation indicates substantial differences in effects elicitable from epicardial pores and other spaces, e.g. that of the coronaries.

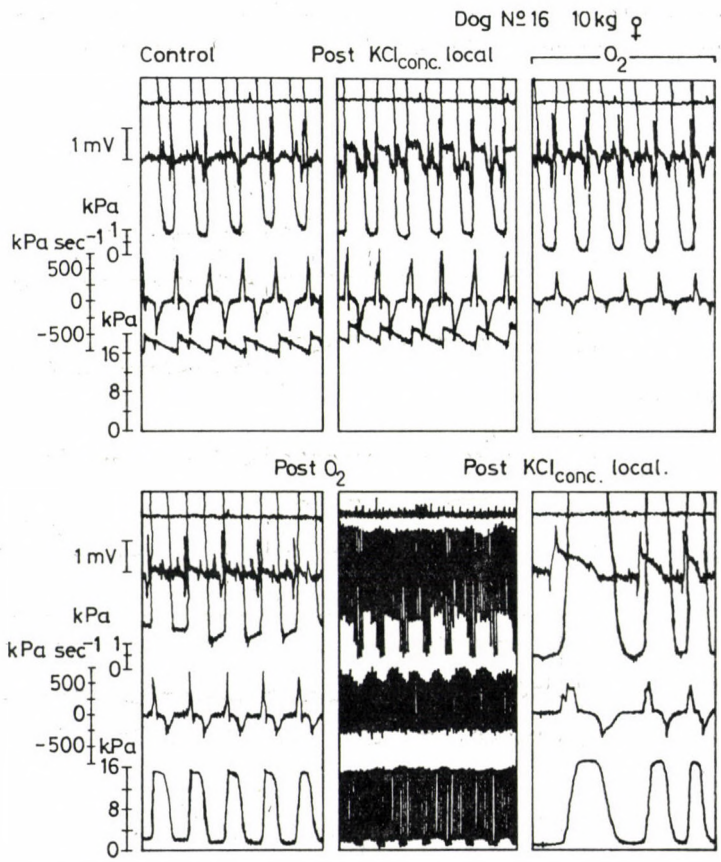
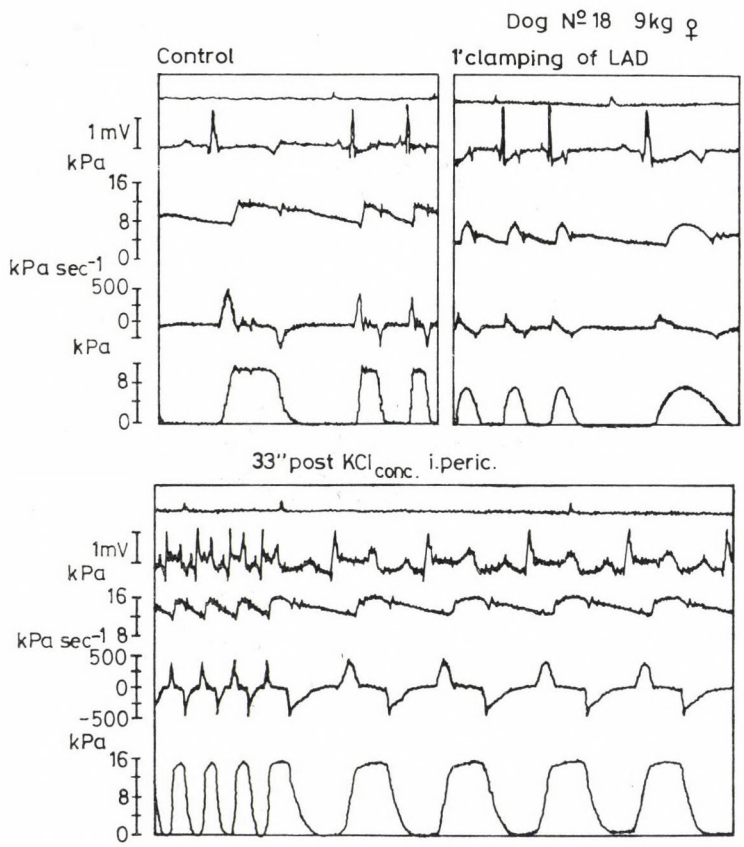


Fig. 6. Effect of oxygenation of the epicardial surface on the changes in ECG and left ventricular end-diastolic pressure evoked by successive application of hypoxia and KCl. Signs from above: ECG lead II, left ventricular end-diastolic pressure, dP/dt, aortic pressure. In the lower part of the Figure the last tracing represents left ventricular pressure. Time: 1 s. First column: control. Second column: 39 s after local administration of KCl_{conc} ST-elevation (dome). Third column: end-diastolic pressure decreases during surface oxygenation and the dome disappears. Lower part: the increase in the left ventricular end-diastolic pressure re-appears after discontinuing surface oxygenation (first and second columns). KCl causes again dome ECG (third column)

As shown by Fig. 8, the effects of hypoxia and KCl could be antagonized by application of the same substance. It can be seen that 19 s after respiratory arrest blood pressure shows an immense elevation. Eighty-three s after the arrest an ST-elevation takes place. The i. peric. administration of substance X inhibits the hypoxic changes both in blood pressure and ECG 4 s and 52 s after respiratory arrest. The ECG remains normal and blood pressure fails to increase. The site of action of KCl and hypoxia is, therefore, the same.

Experiments in rats and guinea-pigs

To learn if the changes observed are only of short duration or represent the beginning of chronic pathological processes, hearts of control and KCl-



Fjg. 7. Comparison of the effects of coronary clamping and KCl. A fall of blood pressure can only be seen after coronary clamping. ST-elevation (lower part) can be seen only after KCl administration as opposed to a decline of ST after clamping of LAD. Tracings: ECG lead II, aortic pressure, dP/dt, left ventricular pressure. Time: 1 s

treated rats were subjected to histological investigation days or weeks after the experiment. Following the acute effects of KCl after 3–5 days an infarction developed; the acute appearance of the monophasic action potential was the triggering process (Figs 9 and 10).

The special staining used for detection of hypoxia showed a marked reaction immediately after the administration of KCl. By days 4–5 following infarction development the staining became weaker but the hypoxic area showed a more diffuse distribution.

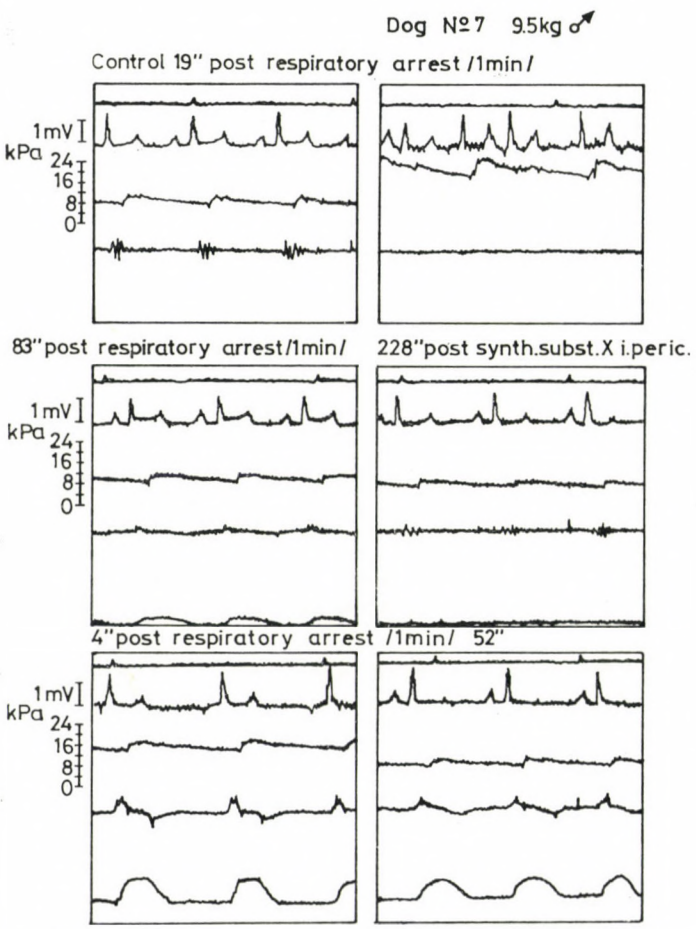


Fig. 8. Hypoxia and KCl-blocking agents. Control period; 19 s and 83 s after the end of hypoxia, respectively; 228 s following the i.peric. administration of substance X; 4 s after the end of the repeated respiratory arrest; 52 s after the end of the second respiratory arrest. Tracings: ECG lead II, aortic pressure, dP/dt. In the lower part the last record is the left ventricular pressure. General hypoxia (asphyxia) in the open-chest animal was induced by switching off the respirator. Elevation of blood pressure and of ST-segment was prevented by the administration of substance X (two lowest records). Time: 1 s

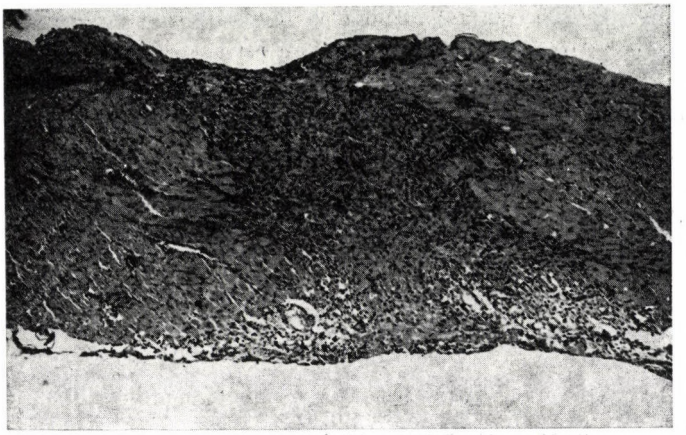


Fig. 9. Large infarction of the right ventricular wall. Haematoxylin-eosin staining. Magnification ×250. (Infarcts were placed mainly in the left ventricle)

Table I

Effect of various substances on dogs' circulation

Substance	Changes in BP				
		systolic		diastolic	
	n	kPa	%	kPa	%
KCl (31.2%)	15	+0.44±0.49	+1.17±2.95	−0.13±0.60	−2.63±4.48
KCl (31.2%) (vagal — excitation)	7	−5.26±0.57 ***	−30.77±1.66 ***	−5.66±0.85 ***	−43.84±3.73 ***
KCl (10%)	5	−0.64±1.00	−4.59±6.39	−0.61±1.17	−5.44±8.93
Synth. subst. X	6	+0.18±0.60	+1.90±3.17	+0.40±0.64	+4.14±4.74
Oxygenation	4	+0.87±0.72	+7.39±6.06	+0.77±0.73	+8.90±8.40

Substance	Changes in LVP			Changes in dP/dt	
	n	kPa	%	kPa · s^{-1}	%
KCl (31.2%)	14	+0.63±0.42	+2.44±2.64	−8.79±22.41	+2.64±3.87
KCl (31.2%) (vagal — excitation)	4	−5.43±0.38 ***	−32.03±0.98 ***	−205.00±46.60 **	−38.90±5.70 ***
KCl (10%)	5	−0.08±0.69	−0.83±4.30	+12.30±33.44	−0.17±6.54
Synth. subst. X	6	−0.09±0.56	+0.22±2.93	−10.25±25.25	−0.71±5.03
Oxygenation	3	+1.69±1.20	+12.3±9.74	+47.83±80.57	−0.95+4.45

**: $p < 0.01$ (paired *t*-test).
***: $p < 0.001$ (paired *t*-test).

Oxygen was led from an oxygen-flask through a needle introduced into the pericardial sac parallel to the myocardial surface. Thick needles served to let the gas out of the pericardial sac. In other cases pieces of filter paper 18–22 mm in diameter were soaked in solutions containing the substance to be studied and applied on the epicardial surface of the heart. In such cases oxygen was applied to the same surface area. Vagal excitation was indicated by the development of a negative inotropic effect together with a decreased heart rate.

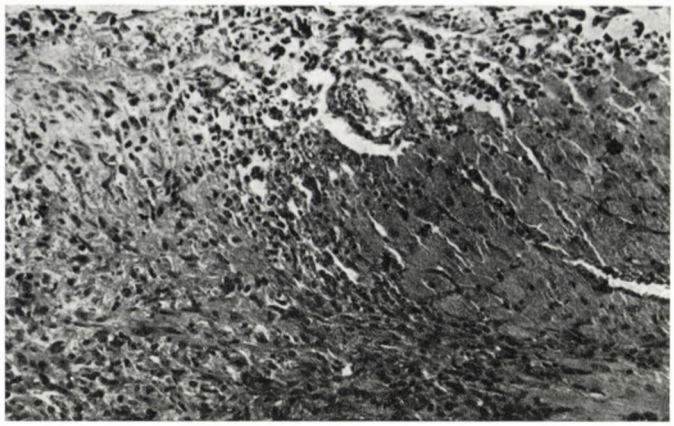


Fig. 10. Part of the infarcted area shown in Fig. 9. Magnification ×600

The KCl-trigger manifests itself in a thickening of the cardiac vasculature. Figure 11 demonstrates that in the hearts of animals 4–5 days after KCl administration the blood vessel index became elevated and in some animals hypertension also developed. The blood vessel index was shifted to higher diameter values. This again means that a phenomenon formerly thought to be mainly of coronary origin can be elicited from the space beginning by the epicardial pores.

Table II

Duration of KCl-evoked ST-elevation (dome) after administration of varios substances in the dog

Substance	Mode of administration	n	T/2 (time elapsed until the reduction of ST-elevation by 50%)	
			s	Δ%
KCl (31.2%)	i. peric.	10	262.5±84.5	—
	local	13	146.2±32.6	—
KCl (10%)	i. peric.	4	115.0±49.2	—
	local	3	115.7±52.5	—
Pericard. samples following KCl (31.2%)		8	32.0± 8.1	−78.28± 7.57***
Synth. Substance X following KCl (31.2%)		6	24.8± 8.7	−68.67±12.07**

**: $p < 0.01$ (paired *t*-test).
***: $p < 0.001$ (paired *t*-test).

T/2 values measured after KCl administration and the effects of natural substances and of synthetic substance X are shown. The time (in s) necessary to reduce by 50% the peak value of the change was taken as T/2. With inhibitory substances, the time elapsed until the fall of ST-elevation to 50% of the value evoked by KCl was measured. The inhibitory substances brought about a significant reduction of the T/2 values. The — values represent the mean values of T/2 of ST-elevation evoked by KCl compared to that appearing after administration of an inhibitory substance measured in the same experiment. Note: in some experiments the protecting substance was given prior to KCl: in these cases the characteristic ECG-effect of KCl could not be observed.

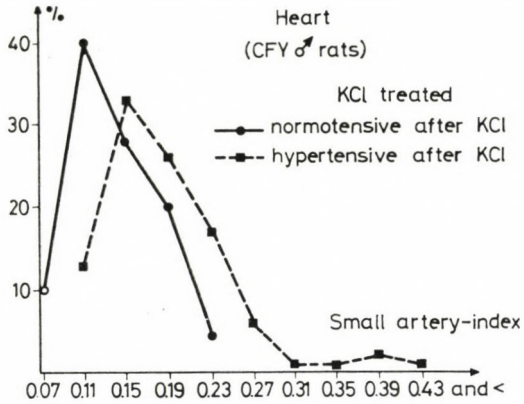


Fig. 11. Changes in vascular index following KCl administration; heart. Abscissa: values o the vascular index. Ordinate: percent incidence of the vascular index values.

$$\text{Vascular index} = \frac{\text{thickness of the media}}{\text{distance between the two external elastic laminae}}$$

Table III

Effect of oxygenation on the increase in blood pressure evoked by general hypoxia (asphyxia)

	n	Increase in systolic blood pressure	
		kPa	%
1. Without oxygen	4	9.20±1.79*	51.85±10.49*
2. During oxygenation	4	2.87±0.58*	16.47± 3.57*
Difference (2−1)	4	−6.33±1.33*	−35.38± 7.86*

	n	Increase in diastolic blood pressure	
		kPa	%
1. Without oxygen	4	4.60±1.19*	29.07±10.36
2. During oxygenation	4	1.60±0.65	11.49± 4.66
Difference (2−1)	4	−3.00±0.56*	−17.58± 6.75

	n	Increase in left ventricular pressure	
		kPa	%
1. Without oxygen	4	9.32±1.72*	53.96±12.24*
2. During oxygenation	4	2.87±0.64*	16.64± 3.59*
Difference (2−1)	4	−6.45±1.10**	−37.31± 8.76*

*: $p < 0.05$ (paired *t*-test).
**: $p < 0.01$ (paired *t*-test).

General hypoxia was induced in open-chest dogs by switching off the respirator. Oxygenation was carried out by i. peric. or direct epicardial application. In both cases, oxygen reduced the degree of blood pressure elevation caused by general hypoxia.

The appearance of infarction in rats depended on the mode of administration of KCl. If KCl was administered into the apical region intrapericardially, the incidence of infarction in animals sacrificed up to day 9 and after day 28 was 45.5% and 55% (n = 46 and 21), respectively. In the case of basal i. peric. administration the incidence proved to be 89% (n = 9).

The incidence of infarction grew to 90% if KCl administration was carried out after thoracotomy, without artificial respiration thus indicating that in this case the initial effect of KCl and of hypoxia were additive (n = 6).

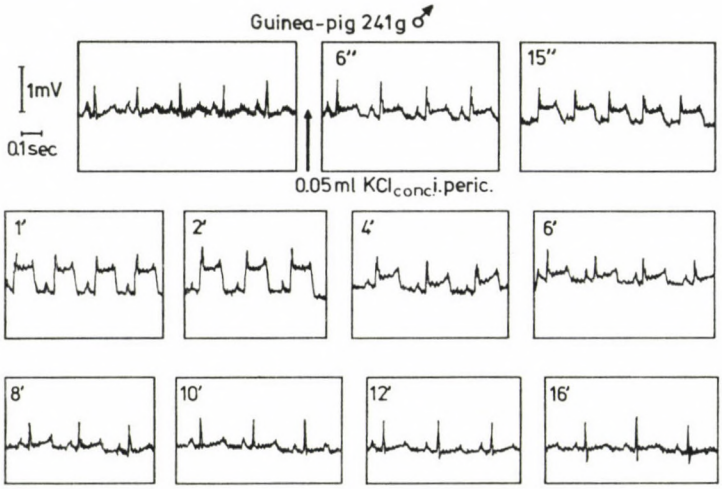


Fig. 12. Effect of 31.2% KCl solution in a guinea-pig (ECG lead II)

Acute influence of KCl on the ECG was essentially the same in rats and guinea-pigs as in dogs. The development of monophasic action potential in guinea-pigs is similar to that observed in dogs (Fig. 12). In this case the ECG change disappeared after 16 minutes. The same result was observed in rats.

No such change could be observed after the injection of concentrated glucose solution or after Ca^{2+} or Na^{+} administration, although in the latter case a reduction in amplitude of the R-wave could be observed. An intravenous injection of KCl evoked the well-known ECG changes of flutter and fibrillation.

As in dogs, also in rats and guinea-pigs some protecting substances appearing in the pericardial fluid prevented the acute effect of KCl; the dome-shaped ST-segment disappeared some seconds or minutes after the administration of pericardial fluid. Figure 13 shows that in rats i. peric. administration of dog pericardial fluid inhibited the development of the dome-shaped ST-segment.

Figure 14 demonstrates the changes in height of the ST-segment evoked by KCl in the rat heart and the statistically significant inhibition of this change caused by i. peric. administration of dog pericardial fluid. This means that the pericardial fluid has a protective effect also in the rat heart.

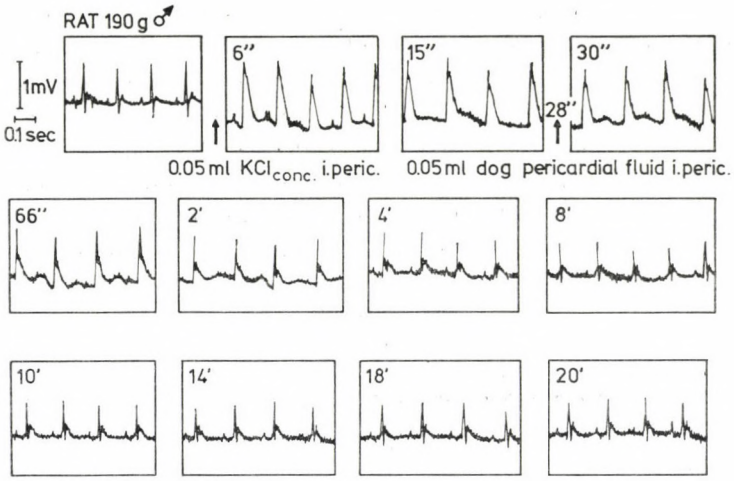


Fig. 13. Rat ECG. Changes evoked by KCl have normalized 4 min after i.peric. administration of dog pericardial fluid

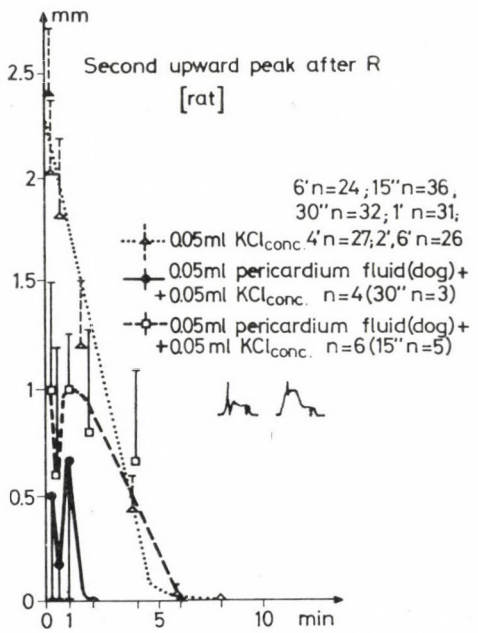


Fig. 14. Height (mm) of ST-segment after KCl and the influence of dog pericardial fluid. Dots and squares represent two different groups of animals. In the first group (squares) the effect of the pericardial fluid was not marked as it was demonstrated on other parts of the ECG curve (not shown in the Figure). Dots represent a group where a maximum normalization of all ECG waves could be observed. The two smaller curves, in the middle of the Figure, visualize the most frequent changes in the ECG curve after KCl the magnitude of which represent the basis of this drawing

A dilute (1%) solution of KCl exhibited a similar protection against the noxious effect of concentrated KCl solution, thus indicating that a small amount of KCl might release the same protective substance which is contained in the pericardial fluid.

The synthetic substances X, Y and Z had the same effect in rats and guinea-pigs as in dogs. As shown in Fig. 15, substance Z almost completely normalized the KCl-evoked ECG changes some 90 sec after its application, while 10 min later the ECG was quite similar as the control curve.

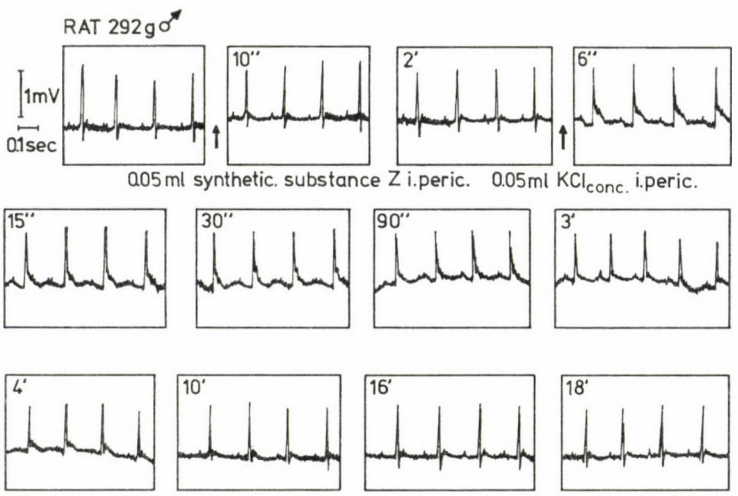


Fig. 15. Protective effect of substance Z. ECG dome disappears 4 min after i.peric. administration of substance Z

Next it was studied whether an inhibition of the acute KCl-induced ECG changes may prevent ensuing infarction. To this end, some animals acutely injected with KCl were sutured after operation and sacrificed 1 to 28 days later. Another group of animals was treated not only with KCl but also with either biological or synthetic protecting substances. Histological investigation showed that these substances reduced the incidence of infarction caused by KCl from 52.4% to 7.14%. In other words, the infarction eliciting effect of KCl was inhibited.

Provided that the above-mentioned space through which KCl application and hypoxia eventually cause infarction in fact begins with pores, the physical obstruction of these pores should prevent the KCl effect. In the experiment shown in Fig. 16 the pores were obstructed by means of India ink. It can be seen that KCl was much less effective than otherwise: the usual dome-shaped ECG change was missing and within 5 min the ECG became normal. The same phenomenon could be observed in the dogs, too.

Table IV demonstrates the statistical evaluation of these data. The maximum effect was achieved after 31.2% KCl solution. The effect of more dilute (10% and 1%) KCl solutions was significantly lower. Following India ink administration, the changes in S and T waves evoked by 31.2% KCl became significantly (unpaired *t*-test) smaller, indicating that the obstruction of the assumed pores prevented the development of KCl evoked changes. Administration of pericardial fluid also prevented the effect of concentrated KCl solution.

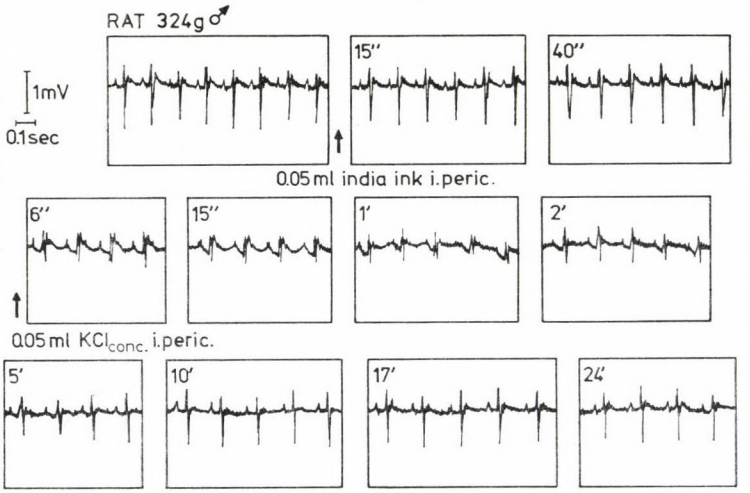


Fig. 16. After obstructing the epicardial pores with India ink (i.peric. administration) KCl fails to affect the ECG

Table IV

Maximum changes (Δ%) in amplitude of ECG waves after i. peric. administration of different substances in 0.05 ml volume in rats

	First minimum after the R-peak (S)			First upward peak after the R-peak (T)		
	n	Δ%	p	n	Δ%	p
KCl_{conc} (31.2%)	24	−363.98±24.64		36	+441.36±76.63	
KCl (10%)	6	−177.33±33.18	<0.01	5	+123.34±52.27	<0.01
KCl (1%)	11	−72.26±16.93	<0.001	11	+49.25±22.71	<0.001
India ink + KCl_{conc}	5	−141.40±31.35	<0.001	5	+113.00±37.67	<0.001
Pericard. samples + KCl_{conc}	3	−174.33±16.73	<0.02	3	+64.43± 9.87	<0.001

p denotes the significance level of differences from values measured after 31.2% KCl administration (unpaired *t*-test).

The effect of KCl in rats is the same as in dogs and can be inhibited similarly with natural and synthetic substances. In these experiments the protective substances were administered prior to KCl administration. intrapericardially, and the changes in S and T waves were measured.

The effect of KCl has been given in changes of the S and T waves as compared to the control values before KCl administration.

Discussion

The present studies have shown that the infarction and its accompanying changes may originate in a special space of the heart, since i. peric. injection of KCl produced a monophasic action potential similar to that observed in human infarction and 3–5 days later an infarction really ensued. This myocardial space is probably distinct from that supplied by the coronary blood vessels, since intravenous K^+ administration leads to the well-known phenomena of flutter and fibrillation, while KCl introduced into the pericardial space produces a monophasic potential in the ECG characteristic of infarction. The fact that the latter ECG change could be evoked by K^+ applied locally to a small epicardial surface indicates that a similarity exists between the above-mentioned experiments and human infarction-genesis. In both cases infarction may develop in a circumscript area.

Epicardial application of KCl may result also in thickening of the coronary vessels after some days. This finding points to the ability of excess KCl acting in the epicardial space to induce changes in the coronary arteries themselves. Nevertheless, in the majority of cases infarction developed without any changes in the coronary tree. Hence, *the thickening of these arteries is the result of the chain of events eliciting infarction rather than its cause.*

There are several lines of evidence in favour of the concept that KCl evoked the ECG changes characteristic of human infarction through an effect exerted in a separate myocardial space. KCl exerted different effects depending on the mode of its administration. Typical infarction-like ECG record could be elicited through the epicardial space, while i.v. KCl elicited only the well-known flutter and fibrillation. Some agents such as noradrenaline do not act on the heart through the epicardial pores. Conversely, natural and synthetic substances when given into this space afforded protection against the KCl-induced ST-elevation and later infarction. That this space begins with pores, could be inferred from the finding that almost 10% of the ^{125}I-labelled albumin applied to a small epicardial area could be found in the endocardium and in the myocardial fibres beneath it [8]. By obstruction of these pores by means of India ink, a prevention of the above-mentioned effects of i. peric. KCl could be achieved.

The fact that some natural substances protecting against infarction could be found in the pericardial fluid underlines the importance of the pericardial space where the lack of these substances may lead to infarction. The effectiveness of these substances might also indicate the presence of receptor-like structures in this area.

Successful oxygenation of the myocardium via this space fits in well with the described phenomena. Rises in blood pressure and alterations of ECG and of end-diastolic pressure produced either by general hypoxia or by coro-

nary clamping could be prevented by oxygen applied locally on the surface of the heart or introduced into the pericardial sac.

Neither the effect of oxygen nor that seen after KCl administration can be explained by diffusion into the myocardium, this mechanism of transport being much slower than the time-course of the observed changes. The high coronary blood flow and myocardial lymph circulation would eliminate the solution before reaching the myocardium, if the transport of substances from the pericardial space did not occur in a space distinct from the former two circulatory channels. Earlier experiments showed the ability of ^{125}I-labelled albumin to accumulate at an endocardial area opposite to the site of its pericardial application [8]. This fact excludes a sideward diffusion and supports the existence of preformed pathways inside the heart, perpendicular to the endocardium. This assumption was further strenghtened by the present results obtained after occluding these pores with India ink.

These considerations make it unlikely that oxygen applied to a small epicardial area might increase the oxygen content of the hypoxic arterial blood perfusing the heart. Until the results of our experiments now in progress will be available, the most feasible explanation may be the oxygenation of some reducible substrates during hypoxia.

The similar role of KCl and hypoxia in eliciting the circulatory changes and alterations in the membranes are supported by other facts as well. Accordingly, KCl administration evokes hypoxia and the hypoxia leads to an increase in KCl concentration. This correlation is indicated by the finding that the same protective agent is capable of inhibiting the ECG effect of both KCl and hypoxia. *It should be pointed out that KCl and hypoxia evoke infarction from a special space of the myocardial cells. This space begins with pores at the epicardial surface and reaches myocardial cells accross special pathways of connective tissue* (Fig. 17).

The efficacy of this system is shown by the almost 100% incidence of ST-elevation after administration of KCl. Since the pericardial fluid was shown to contain biologically potent substances, a branching of the intramyocardial canalicular system towards the surface of the heart has been supposed. Active substances may influence the myocardial cells either via the epicardial pores or through the coronary circulation. Figure 17 also demonstrates that an excess of K^+ evoked by hypoxia may re-enter the pores, aggravating thereby the existing hypoxia which, in turn, leads to a further accumulation of K^+. This K^+ — hypoxia feed-back mechanism finally results in infarction. Although in our experiments K^+ had a triggering role, the same situation after hypoxia may also be assumed. The latter cause-effect relationship is supported by our finding that when KCl was administered after opening the chest (rats), hypoxia was added to the K^+ effect, thus, the incidence of infarction reached 90%. The site of KCl injection also influenced the incidence of infarction; injection

at the base and at the apex resulted in infarction in 89% and 50%, respectively. In humans, too, infarctions are more frequent at the base than around the apex of the heart.

The separation of the two spaces seen in Fig. 17 does not mean that the epicardial space is supplied with oxygen from a system other than the coronaries; but the epicardial space may represent a supplementary or alternative route for oxygen in the case of hypoxia. Fig. 17 shows further that substances administered into the pericardial fluid ultimately reach the extracellular space.

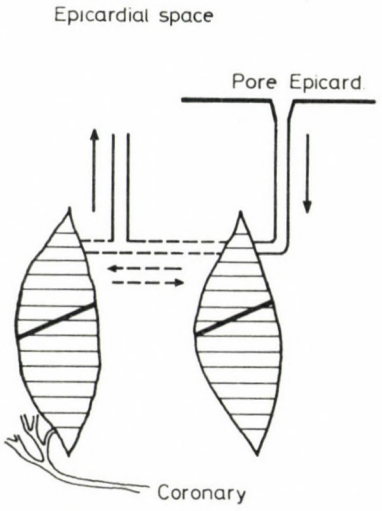


Fig. 17. Schematic diagram (for details see text)

This is supported by a number of data. WEISS and SCHIENE [10] found that during myocardial ischaemia K^+ accumulates in the extracellular space. According to WIEGAND et al. [11] after clamping of the coronary artery extracellular K^+ concentration increases from 3.2 mmol/litre to 11.3 mmol/litre. Similar results have been obtained by HILL and GETTES [6], HIRCHE et al. [7], HARRIS et al. [4,5] as well as by DENNIS and MOORE [2].

It should be emphasized that the ECG changes observed in the present experiments are very similar to those known to develop in man in the course of infarction. The initial rise of the ST-segment, disappearing during infarction, may re-appear at later stages as observed also by DIETRICH and SCHWIEGK [1]. Inhalation of hypoxic gas mixtures led to re-appearance of ST-elevation as did exercise (HAUSNER and SCHERF, [1]). Release of K^+ was observed also by FRANZ and BÖS [3] during myocardial ischaemia. Thus, the majority of data available are not in contradiction with our theory of the development of myocardial infarction.

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THE EFFECT OF DEOXYCORTICOSTERONE ON THE INTERSTITIAL FLUID PRESSURE IN NORMAL RATS

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Interstitial fluid pressure was measured by Guyton's capsular method in the subcutaneous tissue of rats after a single DOC i.p. injection. Regardless of the dose of DOC, the pressure-time curve showed three phases: latency, decrease and recovery. The maximum drop in pressure and the recovery time were dose-dependent. Maximum decrease in interstitial fluid pressure after 0.25; 0.50; 0.75; 1.00 mg/100 g body wt of DOC was 3.2; 7.1; 9.2 and 10.6 mmHg, respectively. It was found that the recorded pressure response is not exclusively caused by Starling forces. A correlation was found between the pressure decrease and the extracellular potassium loss. The role of potassium in the DOC-induced pressure response was verified by administration of the specific potassium ionophore, valinomycin. Due to the effect of valinomycin, potassium ion transport and, as a consequence, the interstitial pressure response, were accelerated.

GUYTON [6] reported that interstitial hydrostatic pressure in the subcutaneous space was normally subatmospheric when measured with chronically implanted capsules. Some studies [6, 7, 8, 13] supported that the interstitial fluid pressure was normally negative in certain soft tissues. In all these experiments alterations in interstitial hydrostatic pressure were induced by changing the hydrostatic pressure in the capillary or the colloid osmotic pressure of plasma, and the measured interstitial pressure changes corresponded to those predicted by STARLING's law [11].

Recently, in the development of negative capsular pressure more importance has been attributed in addition to the STARLING forces to the physical and chemical properties of the components of the interstitial space [2, 5]. Interstitial proteoglycans are thought to generate an absorption pressure primarily because of the Donnan osmotic pressure of the cations entrapped by these polyelectrolytes and because of the electrostatic repulsion between the negative charges on the proteoglycan molecules themselves. These pressures represent ionization-dependent components of the matrix's (capsular) pressure.

Thus it might be expected that the interstitial pressure may alter due to electrostatic repulsion if the ionic balance and thereby the degree of ionization are influenced. It is known that aldosterone and 11-deoxycorticosterone have marked effects on sodium and potassium distribution in the body. The aim of the present study was to check whether the changes in ionic balance induced by DOC administration do or do not influence the interstitial (capsular) pressure.

Materials and Methods

Experiments were performed on female rats weighing 200–250 g. The animals had free access to food and water prior to study and were maintained under conditions of controlled lighting (from 6 a.m. to 6 p.m.) and temperature (26 ± 2 °C). Anaeshtesia was induced by i.p. injection of sodium pentobarbital 5 mg/100 g body wt during measurements. Supplemental doses of sodium pentobarbital were given as required to maintain anaesthesia.

Interstitial fluid pressure measurement. The interstitial fluid pressure was determined by GUYTON's method [6]. A cylindrical plastic tube 5 mm in diameter and 15 mm long, closed at both ends and perforated by numerous 1 mm holes was implanted subcutaneously in the back of rats 4–6 weeks before the experiments. The actual pressure prevailing in the inner space of the capsule was measured by a 26 gauge syringe needle inserted through the skin, and through one of the holes into the cavity of the capsule. The needle was connected to a Statham transducer (P 23 Db) by polyvinyl tube. The whole system was previously filled with normal saline solution.

Analytical methods. Samples of blood from a carotid artery and of interstitial fluid from the capsule were collected in heparinized tubes and centrifuged immediately thereafter. Sodium and potassium concentrations were determined by flame photometry (Flapho 4, Zeiss). Total protein concentration was measured by the biuret method, using bovine serum albumin as standard. Free fatty acids were assayed by the DOLE and MEINERTZ method [3] and glucose by DUBOWSKI's method [4]. All these measurements were performed in both plasma and interstitial fluid samples collected simultaneously.

Other determinations. Mean arterial blood pressure was measured by a catheter inserted into the carotid artery and connected to a mercury manometer. Arterial haematocrit was measured using a micro method. In all experiments measurements of arterial pH, pCO_2 and pO_2 were carried out using a "IL" 213-227 digital pH/blood gas analyser.

Drugs. A single injection of desoxycortone acetate in oil (Decosteron, Richter) or water-soluble desoxycortone glucoside (Percorten, CIBA) was given intraperitoneally in doses of 0.25; 0.50; 0.75; and 1.00 mg/100 g body wt.

Statistical analysis. All experimental data are presented as means ±S.E.M. Statistical significance was determined by Student's *t*-test; $p < 0.05$ was considered significant.

Results

a) *Effect of DOC on ionic balance, blood pressure, pH, blood gases, protein, glucose and FFA*

The well-known effects of mineralocorticoid excess were detected following administration of 1 mg/100 g body wt DOC. The relative sodium concentration was significantly increased in both plasma and capsular fluid. While reduction of the relative potassium concentration in the plasma was pronounced, in capsular fluid it was slight (Fig. 1). An ion shift between plasma and capsular fluid was not observed in the control animals (Table I).

Besides hypernatraemia and hypokalaemia, hypertension and compensated metabolic alkalosis (see Table II) were also observed.

The total protein concentration was higher than the control in both plasma and capsular fluid. The increase in plasma free fatty acids (FFA) and glucose concentrations pointed to some glucocorticoid activity of desoxycortone glucoside (Table III).

Table IV shows the difference between the interstitial and plasma protein concentration (called osmotically effective protein concentration). The effective protein concentration reflects only the direction of changes of the colloid

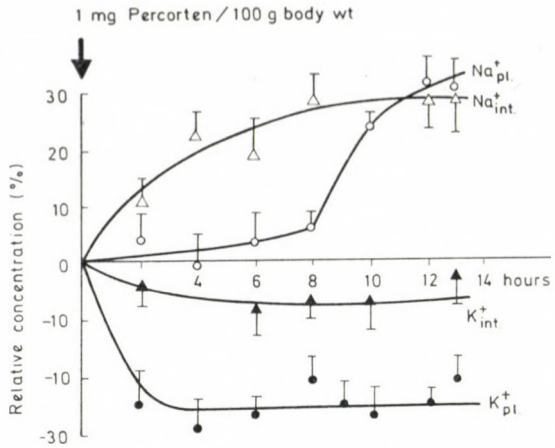

Fig. 1. Sodium and potassium concentrations in plasma and capsular fluid following i.p. administration of 1 mg/100 g of desoxycortone glucoside expressed in percents of control. Each point is the mean ±S.E.M. for 8–10 different animals

Table I

Control sodium, potassium and protein concentrations (means ± S. E. M.) in plasma and capsular fluid immediately and 8 h after anaesthesia

Parameter		Control I	Control II	p, I vs II
Sodium concentration mmol/l	plasma	143 ± 1 (n = 7)	141 ± 1 (n = 7)	N. S.
	capsule	145 ± 1 (n = 7)	144 ± 2 (n = 7)	N. S.
p, plasma vs capsule		N. S.	N. S.	
Potassium concentration mmol/l	plasma	4.9 ± 0.3 (n = 7)	5.2 ± 0.2 (n = 7)	N. S.
	capsule	5.4 ± 0.2 (n = 7)	5.7 ± 0.2 (n = 6)	N. S.
p, plasma vs capsule		N. S.	N. S.	
Protein concentration, g/l	plasma	67.94 ± 1.74 (n = 7)	64.58 ± 2.72 (n = 7)	N. S.
	capsule	41.13 ± 1.79 (n = 6)	41.70 ± 1.46 (n = 8)	N. S.
p, plasma vs capsule		<0.001	<0.001	

Control I: immediately after anaesthesia.
Control II: 8 hours after anaesthesia.
p, I vs II: significance of differences between Control I and Control II.
p, plasma vs capsule: significance of differences between plasma and capsular (interstitial) fluid.

Table II

Mean values ± S. E. M. of blood pressure, pH, pCO_2, pO_2 and haematocrit for arterial blood after a 1 mg/100 g dose of desoxycortone glucoside

Parameter		Control	Hours after injection of 1 mg/100 g of desoxycortone glucoside		
			4	8	12
Blood pressure mmHg		119 ± 3 (n = 8)	114 ± 4 (n = 4)	136 ± 5* (n = 4)	145 ± 5* (n = 6)
pH		7.28 ± 0.02 (n = 8)	7.32 ± 0.04 (n = 7)	7.32 ± 0.02 (n = 8)	7.37 ± 0.03 (n = 8)
pCO_2	mmHg	45.4 ± 1.8	43.2 ± 2.3	38.2 ± 1.2*	40.1 ± 1.7
	kPa	6.04 ± 0.24 (n = 8)	5.75 ± 0.31 (n = 6)	5.08 ± 0.16* (n = 8)	5.33 ± 0.23 (n = 8)
pO_2	mmHg	88.3 ± 8.2	84.3 ± 2.5	88.2 ± 2.8	86.2 ± 1.3
	kPa	11.74 ± 1.09 (n = 8)	11.21 ± 0.33 (n = 7)	11.73 ± 0.37 (n = 8)	11.46 ± 0.17 (n = 8)
Haematocrit		44 ± 0.4 (n = 15)	44 ± 1.0 (n = 13)	44 ± 0.8 (n = 15)	45 ± 0.9 (n = 10)

*: $p < 0.01$, significantly different from controls.

osmotic pressure. The change in this gradient referring to the control state $\Delta(c_i - c_{pl})$ follows the plasma osmotic pressure shift after DOC injection. From the third column of Table IV it can be seen that the $\Delta(c_i - c_{pl})$ was unchanged, except in the 4th and 6th h of the experiment when it was increased.

Table III

Plasma protein, FFA and glucose concentration (means ± S. E. M.) after administration of 1 mg/100 g desoxycortone glucoside

Parameter		Control	8 h after injection of 1 mg/100 g desoxycortone glucoside	p
Protein, g/l	plasma	65.3 ± 1.5 (n = 9)	84.1 ± 2.0 (n = 9)	<0.01
	capsule	42.2 = 1.3 (n = 9)	54.0 ± 3.6 (n = 9)	<0.01
FFA, mmol/l	plasma	1.01 ± 0.16 (n = 9)	2.22 ± 0.33 (n = 9)	<0.01
Glucose, mmol/l	plasma	7.10 ± 0.33 (n = 9)	8.60 ± 0.22 (n = 9)	<0.05

p: significantly different from controls.

Table IV

Effective protein concentration between capsule and plasma after a 1mg/100 g dose of desoxycortone glucoside

Hours	$c_i - c_{pl}$ g/l	$\Delta(c_i - c_{pl})$ g/l	p
0	-25.8 ± 2.8	0	
2	-21.6 ± 8.0	$+4.2$	N. S.
4	-13.8 ± 2.5	$+12.0$	<0.05
6	-11.9 ± 1.3	$+13.9$	<0.02
8	-31.3 ± 5.0	-5.5	N. S.
10	-30.8 ± 0.4	-5.0	N. S.
12	-21.4 ± 4.4	$+4.4$	N. S.
13	-16.5 ± 4.2	$+9.3$	N. S.

c_i: total protein concentration in interstitial (capsular) fluid (n = 5).
c_{pl}: total protein concentration in plasma (n = 5).
$\Delta(c_i - c_{pl}) = (c_i - c_{pl})_{DOC} - (c_i - c_{pl})_{Control}$.
Plasma and capsular fluid were collected from the same animal.
p: significantly different from control.

b) *Effect of DOC on capsular pressure*

In Fig. 2 the changes in interstitial fluid pressure following an injection of 1 mg/100 g body wt of desoxycortone glucoside is represented by a typical curve. The control pressure was —4.7 mmHg. After desoxycortone glucoside administration a latency period of 3 h occurred then the pressure started to fall progressively and subsequently it returned to the control value. The shape of the pressure curve remained the same irrespective of the dose of DOC, i.e. the 2–3 h latent period and the slope of pressure decrease were almost iden-

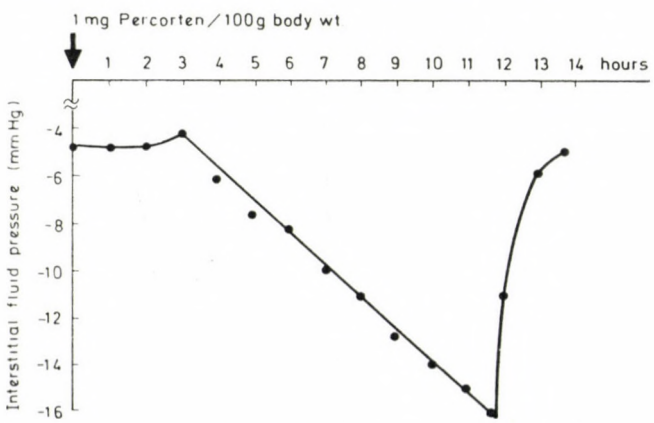

Fig. 2. Effect of 1 mg/100 g body wt of desoxycortone glucoside on interstitial fluid pressure

tical. The maximum interstitial fluid pressure and recovery time were, however, dose-dependent.

In Fig. 3 the maximum decrease in interstitial fluid pressure is plotted as a function of the DOC dose. The drop in interstitial fluid pressure after 0.25;

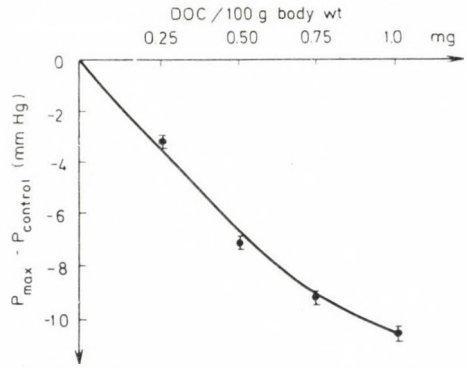

Fig. 3. Effect of DOC in different doses on the maximum interstitial fluid pressure response Each point is the mean value ±S.E.M. for 5 different animals

0.50; 0.75 and 1.00 mg/100 g of DOC was 3.2 ± 0.2 (n = 5), 7.1 ± 0.2 (n = 5), 9.2 ± 0.2 (n = 5) and 10.6 ± 0.3 mmHg (n = 5), respectively.

Figure 4 shows the effect of the potassium ionophore valinomycin on the control pressure and the DOC-induced interstitial fluid pressure response in a typical experiment. Administration of 10 μg valinomycin into the capsule resulted in a slight increase of interstitial fluid pressure above the control level. The addition of valinomycin altered the DOC-induced interstitial fluid pressure response in that the latency period was shortened and the pressure decrease accelerated, but the lowest value of the pressure remained unchanged.

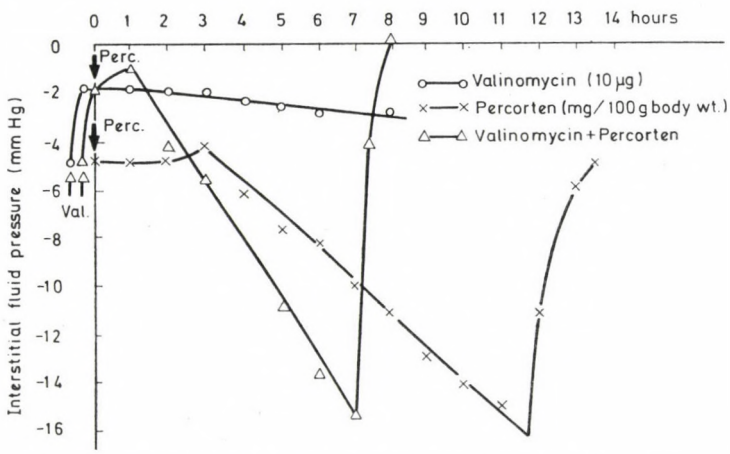

Fig. 4. Effect of valinomycin on the control and the DOC-induced interstitial fluid pressure changes

Discussion

We found significant changes in interstitial fluid pressure after a single DOC i.p. injection. In order to ascertain the exact origin of the observed interstitial fluid pressure response, the influence of volumetric, osmotic and other possible factors operating in the interstitial space had to be analysed.

In the experiments, the fluid volume was not measured directly. The haematocrit and the sodium concentration allowed to draw some conclusions on the possible changes in extracellular volume. The increased sodium reabsorption in the kidney caused by DOC led to a rise in extracellular sodium concentration. Due to the raised osmotic pressure water had to move into the extracellular space. Because no water intake was allowed during the measurements, the relative lack of water in the extracellular fluid could be compensated only at the expense of intracellular fluid.

In the first 8 h period the volume of plasma was probably better preserved than that of the interstitial fluid. This was supported by the practically constant plasma sodium concentration and by the constant haematocrit value. As a result there was an increase in interstitial fluid volume at the expense of intracellular fluid.

In the 8–13 h period, the volume of interstitial fluid increased at the expense of both intracellular fluid and plasma. This hypothesis is supported by (i) the significant increase in sodium concentration both in plasma and interstitial fluid; (ii) the increase in plasma protein concentration, and (iii) the constant haematocrit as a result of two opposite effects: the decrease of plasma volume and the increase of extracellular osmolarity.

There seems to be a contradiction between the increased protein concentration of the interstitial fluid and the increased interstitial fluid volume. The increased protein concentration following DOC injection was not caused by dehydration, because the protein concentration in both plasma and capsular fluid remained unchanged in the control animals during the 0—8 h period of anaesthesia. This finding therefore is considered as a result of DOC administration, without knowing its exact mechanism.

The elevated blood pressure despite the decreased plasma volume in the 8th and 12th h may be a consequence of an increase in endogenous aldosterone secretion via the renin-angiotensin system. Vasopressin secretion may also increase due to the increased effective plasma osmotic pressure. Both mechanisms result in retention of water, consequently a decrease in urine volume. In fact, micturition was never observed during the 13 h experimental period.

Comparing the possible changes in volume of the interstitial fluid with the measured trends in interstitial pressure, it can be seen that when the interstitial fluid volume was increased (in the first 12 h period after DOC administration), the pressure was constant or it decreased. On the other hand, when the

pressure returned to its control value (in the 12th h after DOC administration) the fluid volume was still augmented.

It is concluded that the volume change may contribute to the observed changes in interstitial fluid pressure but this pressure change is not solely a volume effect.

In order to analyse the osmotic influence on the interstitial fluid pressure, the following factors were taken into consideration. We analysed the equilibrium between plasma and capsular fluid, assuming that the composition of interstitial and capsular fluid was identical [1, 12]; and we calculated the osmotic pressure change due to the alteration in protein concentration.

In the first 10 h period, the colloid osmotic gradient was unchanged or increased in the 4th and 6th hours. This contradicts the simultaneous continuous decline of interstitial fluid pressure. No significant change was seen in this gradient during the next 10—13 h period, when the pressure increased. So the trend of interstitial fluid pressure change cannot be interpreted by the osmotic effect of proteins.

The insufficiency of STARLING forces in the interpretation of the DOC-induced pressure decrease allowed to assume that the pressure change may be related to the interstitial ionic equilibrium, above all to the losses of potassium ion. With decreasing potassium concentration the amount of entrapped potassium ions, consequently the number of negative charges on the proteoglycan molecules, and according to this, the electrostatic repulsion increases. Interstitial pressure changes are related to the number of entrapped cations. Thus it is not necessary for the pressure decrease to coincide directly with the decrease in total (entrapped plus free) extracellular potassium concentration. As direct measurements could not be done it was assumed that the fall of interstitial fluid pressure was correlated with the interstitial losses of potassium ion. The hypothesis seems to be supported by the effect of valinomycin [9, 10]. Valinomycin increases the potassium permeability of membranes and so that of the membrane around the capsule. Therefore, due to the effect of valinomycin the potassium ion transport and, as a consequence, the interstitial fluid pressure response is accelerated as it has been observed in the present study.

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BETANINURIA: A GENETIC TRAIT?

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A total of 58 MZ and 41 DZ Hungarian adult like-sexed twin pairs were studied for the presence of betanin in urine (called *betaninuria)* after the ingestion of a standardized beetroot juice. By means of a semi-quantitative technique, betaninuria values of all test persons were determined. Several mathematical models were tested to find a probability distribution fitting well to the empirical data.

The hereditary character of the intensity of betaninuria cannot definitely be accepted although some circumstances pointed to the effect of genetic factors on the phenomenon.

ALLISON and MCWHIRTER [1] described two new traits for which human individuals have been suggested to differ. The first trait is that after eating asparagus some people excrete methyl mercaptan, a compound of unpleasant smell, while others do not; the gas can be identified in urine by transforming it to silver mercaptide. The second trait is characterized by the urinary excretion of a violet-red pigment a few hours after consuming beetroot by some of the people. The pigment was identified as the alkaloid *betanin*, a betacyanin of reddish-violet colour. Its structure was clarified by MABRY et al. [16] (Fig. 1).

The presence of betanin is often confused with haematuria, thus the phenomenon has a practical importance. Early authors, ZINDLER and COLOVOS [26] attributed the presence of betanin in urine to food allergy while ALLISON and MCWHIRTER [1] supposed that *betaninuria* (abbr.: BU) was genetically controlled and that in healthy populations there were excretors and non-excretors, strongly suggesting that the character was transmitted by a single, autosomal recessive gene. Family data were in accordance with their hypothesis, but it must be noted that their method was too simple: the presence or absence of the pigment was registered by a simple inspection of the urine. They found that among 104 persons 10 were excretors; and since their family data seemed to confirm the results, the authors supposed that the phenomenon might have played a role in human selection to the advantage of heterozygotes. An Editorial of the *British Medical Journal* [3] supported the hypothesis, but shortly thereafter it was rigidly refused by PENROSE [18] in the columns of the same periodical, judging the theory as an "inaccurate impression". Later SALDANHA et al. [20, 21, 22] confirmed the results of ALLISON and MCWHIRTER not only by emphasizing the validity of the genetic theory, but also by finding ethnic

differences in BU among Caucasian, Japanese and Negro populations living in Brazil. Their method, however, was rather primitive, excretion or non-excretion was judged by visual criteria. Another hypothesis, introduced by WATSON et al. [24, 25] and LUKE and WATSON [15], supposed that urinary excretion of betanin depended on the degree of intestinal absorption and not on the excretion type of the individual, influenced by sufficient or insufficient denaturation of the compound, as it was assumed by ALLISON and MCWHIRTER [1].

Complete rejection of the genetic theory was expressed by GELDMACHER—v. MALLINCKRODT et al. [12, 13] who investigated BU by column chromatography. It must, however, be mentioned that beetroot ingestion in their cases was not controlled reliably. They concluded that none of the hitherto expressed

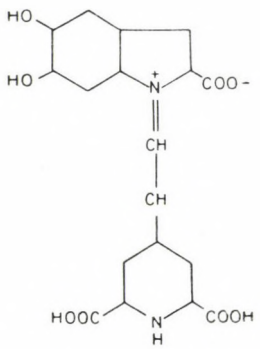

Fig. 1. Structure of betanin

hypotheses could be accepted. According to them, everyone may be regarded as a betanin excretor, it only depends on the amount of the beetroot ingested and the method applied.

The great uncertainty in the literature concerning the detection of BU on the one hand and the aetiological interpretation of the results on the other, had led us to elaborate a new method [6, 7]. In an experiment performed in 244 kindergarten children 3 to 6 years of age, the juice of cooked beetroot was offered as a drink instead of feeding whole beetroot with a variable pigment content. The urine samples were examined by a Pulfrich photometer to allow a *quantitative characterization* of BU.

As to the individual variability of BU (FORRAI et al. [8, 9]), certain regularity was found: there were people who excreted consistently more, and others who excreted consistently less betanin than the average. A study (FORRAI et al. [10, 11]), was then undertaken to check the statement of WATSON et al. [24] and TUNNESSEN and SMITH [23] who found a connection between the degree of BU and iron deficiency; this could not be verified by our results.

Materials and Methods

A study was undertaken at the Department of Physical Anthropology of the Eötvös Loránd University, Budapest, in 58 MZ and 41 like-sexed DZ Hungarian adult twin pairs.

The zygosity diagnosis was mainly based on blood group determinations (according to REX-KISS et al. [19]), with 3 red blood cell membrane systems and 2 serum protein systems). On the basis of this serological screening about 93% of the DZ pairs could be detected. In order to separate the remaining DZ pairs hidden in the original MZ group, morphological features, anthropometric measurements, dermatoglyphic patterns, PTC taste sensitivity etc. were also taken into account; e.g. 6 pairs were left out of the original set of MZ twins, their zygosity being regarded as ambiguous on the basis of their PTC sensitivity.

The test persons were asked to void urine before drinking 250 ml of beetroot juice, and then they were allowed no food or fluid for two and a half hours, when collection of the urine samples took place. As to the preparation of beetroot juice see FORRAI et al. [6].

Since the betanin could not be extracted from urine by means of solvents [6], BU was estimated indirectly by determining the extinction at two wavelengths: at 530 nm (yellow colour of urine together with violet colour of betanin) and at 660 nm (yellow colour of urine alone, where betanin practically ceases to absorb light). By substracting the latter from the former value, the result $\Delta E = E_{530} - E_{660}$ was used to characterize the BU of a test person. In the following the $10^3 \times \Delta E$ quantities (having no dimension) will be called simply *BU values*.

In each pair one of the twins was denoted randomly by A while the other one by B. It will not only be dealt with MZ and DZ pairs, but also with the subpopulations of individuals: MZ/A, MZ/B, DZ/A and DZ/B. The frequency distributions of the sampled BU values are seen in Table I. In order to compare our earlier results obtained in kindergarten children with those for members of adult twin pairs, the former data will be referred to as Children.

Mathematical investigation of the BU values

On the basis of inspection the urine samples can be classified into a few discrete categories. In virtue of the method of evaluation applied by us the obtained BU values can be regarded as results of a sampling from any continuous probability distribution. Therefore the statistical treatment of the sampled data consists mainly of a search for the "best fitting" theoretical distribution.

The frequency distributions belonging to all populations investigated by us suggest that the theoretical distribution might have an *unimodal, skew density function* (see Table I). The fact of skewness is beyond dispute while the distinction between unimodality and multimodality leads to more difficult problems discussed later.

Table I

Frequency distributions of BU values (measured in $10^3 \times \Delta E$ units) for the MZ and DZ twins

BU values	MZ/A	MZ/B	MZ	DZ/A	DZ/B	DZ
0—20	1	2	3	2	1	3
21—40	4	6	10	2	3	5
41—60	10	8	18	7	12	19
61—80	13	19	32	6	9	15
81—100	7	4	11	7	3	10
101—120	13	7	20	3	4	7
121—140	2	5	7	3	2	5
141—160	2	1	3	4	2	6
161—180	2	2	4	2	2	4
181—200	1	1	2	3	2	5
201—220	1	2	3	2	—	2
221—240	1	1	2	—	—	—
241—260	1	—	1	—	—	—
261—280	—	—	—	—	1	1
281—	—	—	—	—	—	—
Total	58	58	116	41	41	82

A trial to fit a normal distribution to the BU values of Children population was performed earlier (FORRAI et al. [6], [7]); it was found that the normal distribution failed to fit the frequency distribution.

In the present paper we investigate the fitting of models connected with the lognormal distribution.

A random variable is lognormally distributed, if its logarithm has a normal distribution. A normally distributed random variable is often interpreted as a sum of a large number of relatively small, independent random fluctuations. In a similar way, a lognormal random variable may be regarded as a product of a large number of independent random fluctuations. For the lognormal distribution see e.g. the book of AITCHISON and BROWN [2].

The choice of the lognormal distribution provides a good fit to the empirical data; besides this choice is supported by the analytical properties of the distribution, in particular by the possibility of treatment of the two-dimensional case applied to modelling the distribution of BU values of the twin pairs.

In order to characterize the distribution of the BU values the following models were studied:

1. The two-parametric lognormal distribution. The obtained results could be regarded as fairly good at the first approximation. Several facts suggested, however, that the use of models containing three or more parameters might provide a better description of the phenomenon.

2. The three-parametric lognormal distribution.

3. A superposition of the two-parametric lognormal distribution and a normal distribution with expectation zero and constant variance; by the latter distribution the measuring errors and/or the individual fluctuations of BU values are modelled.

4. A generalization of Model 3, by taking the variance of the "true" BU values. This assumption was indicated by some numerical results contained in papers [8], [9]; namely, in a small population of kindergarten children tested repeatedly ten times, intraindividual variances seemed to be correlated with the average BU values.

In the following, first the mathematical models and the statistical estimating procedures will be described,* then the analysis of the numerical results and a comparison of the models.

Model 1

a) *Univariate case.* The investigated population consists of N persons. The BU value for any person is supposed to be a random variable ξ having a lognormal distribution with parameters (μ, σ^2), i.e. a density function

$$f(x) = \frac{1}{\sqrt{2\pi}\,\sigma x} e^{-\frac{(\ln x - \mu)^2}{2\sigma^2}} \qquad (0 < x < +\infty). \tag{1}$$

The BU values belonging to different persons are assumed to be independent.

Between the moments and the parameters** of this distribution the following connection exists:

$$\mathrm{E}(\xi^k) = \int_0^{+\infty} x^k f(x)\,\mathrm{d}x = e^{k\mu + \frac{k^2}{2}\sigma^2} \qquad (\mathrm{k} = 1, 2, \ldots); \tag{2}$$

consequently the parameters can be evaluated when any two moments of the distribution are known. Let us introduce the notation

$$m_k = \frac{1}{N}\sum_{i=1}^{N} x_i^k \qquad (k = 1, 2, \ldots), \tag{3}$$

where x_i denotes the observed BU value belonging to the i-th person and m_k is called the k-th empirical moment. On the basis of the sample the value of $\mathrm{E}(\xi^k)$ can be estimated by m_k $(k = 1, 2, \ldots)$, and in this way an estimate

* In this paper the estimation method, called *the method of moments*, was used. A comprehensive treatment of multimodality problems can be found in the book of MEDGYESSY [17].

** μ and σ^2 are the expected value and the variance of ln ξ, respectively.

(μ^*, σ^{*2}) of (μ, σ^2) can be given. E.g. the use of the first two moments leads to the equations

$$\begin{cases} m_1 = e^{\mu^* + \frac{\sigma^{*2}}{2}} \\ m_2 = e^{2\mu^* + 2\sigma^{*2}}; \end{cases} \tag{4}$$

the solution yields the estimates***

$$\begin{aligned} \mu^* &= 2 \ln m_1 - \frac{1}{2} \ln m_2, \\ \sigma^{*2} &= \ln m_2 - 2 \ln m_1. \end{aligned} \tag{5}$$

b) *Bivariate case.* In this case the investigated population consists of N twin pairs. The BU values for the members of any pair are supposed to be a random vector variable ($\xi^{(1)}$. $\xi^{(2)}$) having a bivariate lognormal distribution with parameters (μ, σ^2, r) i.e. a density function

$$h(x, y) = \frac{1}{2\pi\sigma^2 \sqrt{1 - r^2}\, xy} \exp\left\{ -\frac{1}{2\sigma^2(1 - r^2)} [(\ln x - \mu)^2 - \right. \tag{6}$$

$$\left. - 2r(\ln x - \mu)(\ln y - \mu) + (\ln y - \mu)^2] \right\} \quad (0 < x < +\infty,\ 0 < y < +\infty).$$

The parameter r characterizes the within-pair correlation* while the BU values belonging to members of different pairs are regarded to be independent. The parameter r appears in the expressions of the product moments of distribution (6), e.g.

$$\mathbf{E}(\xi^{(1)}\, \xi^{(2)}) = \int_0^{+\infty} \int_0^{+\infty} xyh(x, y)\, dx\, dy = e^{2\mu + \sigma^2 + r\sigma^2}. \tag{7}$$

When the BU data of the twin pairs are denoted by (x_i, y_i) $(i = 1, 2, \ldots, N)$, the empirical moments may be written in the form:

$$\begin{aligned} m_k(x) &= \frac{1}{N} \sum_{i=1}^{N} x_i^k, \qquad m_k(y) = \frac{1}{N} \sum_{i=1}^{N} y_i^k, \\ m_k &= \frac{1}{2}\big(m_k(x) + m_k(y)\big) \quad (k = 1, 2, \ldots), \\ m(r) &= \frac{1}{N} \sum_{i=1}^{N} x_i y_i. \end{aligned} \tag{8}$$

By attaching the equation

$$m(r) = e^{2\mu^* + \sigma^{*2} + r^*\sigma^{*2}} \tag{9}$$

*** Of course, the parameters can be estimated in a more natural way by using the moments of the distribution of ln ξ; the present method was applied to allow comparison with the other models.

to the equation system (4) and calculating the values of m_1 and m_2 on the basis of (8) instead of (3), the solution for μ^* and σ^{*2} is given by (5), while the value of r* can be obtained from equation (9):

$$r^* = \frac{1}{\sigma^{*2}} \left(\ln m(r) - 2 \ln m_1 \right). \tag{10}$$

The correlation coefficient R of the random variables $\xi^{(1)}$ and $\xi^{(2)}$ can be expressed as

$$R = \frac{e^{r\sigma^2} - 1}{e^{\sigma^2} - 1}.$$

This quantity can be estimated from the sample by

$$R^* = \frac{m(r) - m_1^2}{m_2 - m_1^2},$$

or

$$R^* = \frac{e^{r^*\sigma^{*2}} - 1}{e^{\sigma^{*2}} - 1}. \tag{11}$$

The numerical results obtained by the use of Model 1 are listed in Table II. The results related to the Children population originated from grouped data

Table II

Numerical results obtained on the basis of Model 1

Population	N	μ^*	σ^{*2}	σ^*	r^*	R^*
Children	244	4.2572	0.1787	0.423	—	—
MZ/A	58	4.4058	0.2399	0.490	—	—
MZ/B	58	4.3577	0.2540	0.504	—	—
MZ pairs	58	4.3716	0.2574	0.507	0.4077	0.3758
DZ/A	41	4.5053	0.2482	0.498	—	—
DZ/B	41	4.3280	0.3049	0.552	—	—
DZ pairs	41	4.4183	0.2276	0.477	0.4883	0.4537

(the same refers to the subsequent Tables); the results could not, however, be biased by this fact to a considerable extent because the class intervals in the grouping were small (10 units long).

Model 2

a) *Univariate case.* This model differs from Model 1 in fitting a three-parametric lognormal distribution to the data instead of a two-parametric one; i.e. the BU value for any individual is a random variable

* r is namely the correlation coefficient of $\ln \xi^{(1)}$ and $\ln \xi^{(2)}$.

$$\xi(\tau) = \xi + \tau,$$

where ξ is the random variable considered in Model 1 and τ is a constant (the third parameter of the distribution). The BU values belonging to different persons are supposed to be independent and identically distributed with a density function

$$f_\tau(x) = \frac{1}{\sqrt{2\pi}\,\sigma(x-\tau)}\, e^{-\frac{(\ln(x-\tau)-\mu)^2}{2\sigma^2}} \qquad (\tau < x < +\infty). \tag{12}$$

The moments of the distribution of $\xi(\tau)$ can be expressed by those of ξ (cf. (2)) in a simple way:

$$\begin{cases} \mathbf{E}(\xi(\tau)) = \mathbf{E}(\xi) + \tau, \\ \mathbf{E}(\xi^2(\tau)) = \mathbf{E}(\xi^2) + 2\tau\mathbf{E}(\xi) + \tau^2, \\ \mathbf{E}(\xi^3(\tau)) = \mathbf{E}(\xi^3) + 3\tau\mathbf{E}(\xi^2) + 3\tau^2\mathbf{E}(\xi) + \tau^3. \end{cases} \tag{13}$$

Substituting the quantities $\mathbf{E}(\xi^k(\tau))$ by the empirical moments m_k ($k = 1, 2, 3$) from formula (3), an equation system is obtained for the estimates $(\mu^*, \sigma^{*2}, \tau^*)$ of the parameters (μ, σ^2, τ); by introducing the notations

$$\begin{aligned} Q &= e^{\sigma^{*2}}, \quad P = e^{\mu^*}, \\ K &= \frac{m_3 - m_1 m_2}{m_2 - m_1^2} - 2m_1, \end{aligned} \tag{14}$$

this equation system can be written in the form

$$\begin{cases} Q^2(Q+3) = 4 + \dfrac{K^2}{m_2 - m_1^2}, \\ P^2 = \dfrac{m_2 - m_1^2}{Q(Q-1)}, \\ \tau^* = m_1 - PQ^{1/2}. \end{cases} \tag{15}$$

It is easy to see that the first equation of (15) has one and only one such a root in respect of the variable Q which is greater than 1. After this equation has been solved, the values for P and τ^* can be calculated from (15) while $\sigma^{*2} = \ln Q$, $\mu^* = \ln P$ are obtained from (14).

b) *Bivariate case.* The BU values belonging to any twin pair are regarded as a random vector variable $(\xi^{(1)}(\tau), \xi^{(2)}(\tau))$, where

$$\xi^{(i)}(\tau) = \xi^{(i)} + \tau \quad (i = 1, 2),$$

and the density function of the joint distribution of $(\xi^{(1)}, \xi^{(2)})$ is given by (6). In this case the empirical moments are calculated on the basis of (8), while the parameters μ^*, σ^{*2}, τ^* are evaluated from the equation system (15). The product moment of the variables $\xi^{(1)}(\tau)$ and $\xi^{(2)}(\tau)$ can be expressed as

$$\mathbf{E}\left(\xi^{(1)}(\tau)\xi^{(2)}(\tau)\right) = \mathbf{E}(\xi^{(1)}\,\xi^{(2)}) + \tau\left(\mathbf{E}(\xi^{(1)}) + \mathbf{E}(\xi^{(2)})\right) + \tau^2,$$

and thus—by the aid of the empirical product moment $m(r)$, using our former notations and considering (15)—the equation

$$m(r) - m_1^2 = P^2Q(e^{r^*\sigma^{*2}} - 1)$$

can be deduced; hence

$$r^* = \frac{1}{\sigma^{*2}} \ln\left(R^*(Q-1)+1\right), \tag{16}$$

where*

$$R^* = \frac{m(r) - m_1^2}{m_2 - m_1^2}$$

is the estimate of the correlation coefficient R of the variables $\xi^{(1)}(\tau)$ and $\xi^{(2)}(\tau)$.

The numerical results obtained by the application of Model 2 are seen in Table III.

Table III

Numerical results obtained on the basis of Model 2

Population	N	τ^*	μ^*	σ^{*2}	σ^*	r^*	R^*
Children	244	−84.39	5.0635	0.0433	0.208	—	—
MZ/A	58	−35.62	4.7858	0.1322	0.364	—	—
MZ/B	58	−45.09	4.8361	0.1196	0.346	—	—
MZ pairs	58	−40.47	4.8121	0.1257	0.355	0.3907	0.3758
DZ/A	41	−209.15	5.7266	0.0299	0.173	—	—
DZ/B	41	−35.71	4.7369	0.1663	0.408	—	—
DZ pairs	41	−87.91	5.1692	0.0833	0.289	0.4635	0.4537

Model 3

a) *Univariate case.* The investigated population consists of N persons; the BU value belonging to any person is a random variable ζ, which can be expressed as a sum of two independent random variables:

$$\zeta = \xi + \eta \tag{17}$$

* The formula (16) is valid in the case of Model 1 too, merely the calculated value of Q is different; the value of R^* is, however, the same as in Model 1.

where ξ has a lognormal distribution with parameters (μ, σ^2) (i.e. its density function is given by (1)), while η is normally distributed with parameters $(0, \Delta^2)$, i.e. having a density function

$$g(z) = \frac{1}{\sqrt{2\pi}\,\Delta} e^{-\frac{z^2}{2\Delta^2}} \quad (-\infty < z < +\infty). \tag{18}$$

The BU values belonging to different persons are assumed to be independent.

The estimation of the parameters μ, σ^2, Δ^2 can be based on the equation system obtained by expressing the first three moments of the distribution of ζ:

$$\begin{cases} \mathbf{E}(\zeta) = \mathbf{E}(\xi) = e^{\mu + \frac{\sigma^2}{2}}, \\ \mathbf{E}(\zeta^2) = \mathbf{E}(\xi^2) + \mathbf{E}(\eta^2) = e^{2\mu + 2\sigma^2} + \Delta^2, \\ \mathbf{E}(\zeta^3) = \mathbf{E}(\xi^3) + 3\,\mathbf{E}(\xi)\,\mathbf{E}(\eta^2) = e^{3\mu + \frac{9}{2}\sigma^2} + 3\Delta^2 e^{\mu + \frac{\sigma^2}{2}}. \end{cases} \tag{19}$$

Namely, according to the assumption of independence of the variables ξ and η, the equalities

$$\mathbf{E}(\eta) = 0, \quad \mathbf{E}(\eta^3) = 0, \quad \mathbf{E}(\xi\eta) = \mathbf{E}(\xi)\,\mathbf{E}(\eta) = 0,$$
$$\mathbf{E}(\xi^2\eta) = \mathbf{E}(\xi^2)\,\mathbf{E}(\eta) = 0, \quad \mathbf{E}(\xi\eta^2) = \mathbf{E}(\xi)\,\mathbf{E}(\eta^2)$$

hold true.

Substituting the moments $\mathbf{E}(\zeta^k)$ of the distribution by the empirical moments m_k $(k = 1, 2, 3)$ given in (3), a non-linear equation system is obtained for the estimates μ^*, σ^{*2} and Δ^{*2} of the parameters μ, σ^2 and Δ^2, respectively, which can be transformed in the following way:

$$\begin{cases} Q(Q^2 - 3) = \dfrac{1}{m_1^2}\left(\dfrac{m_3}{m_1} - 3m_2\right), \\ \mu^* = \ln m_1 - \dfrac{\sigma^{*2}}{2}, \\ \Delta^{*2} = m_2 - Q m_1^2, \end{cases} \tag{20}$$

where $Q = e^{\sigma^{*2}}$. The first equation of the system (20) is a third-order algebraic one; on the basis of its solution the unknown quantities can easily be evaluated: namely $\sigma^{*2} = \ln Q$ (of course only the root $Q > 1$ is acceptable), while μ^* and Δ^{*2} can be calculated from the second and third equation of (20).

b) *Bivariate case.* It is assumed that the BU values belonging to any twin pair can be characterized by a random vector variable $(\zeta^{(1)}, \zeta^{(2)})$, where

$$\zeta^{(i)} = \xi^{(i)} + \eta^{(i)} \quad (i = 1, 2);$$

the density function of the joint distribution of $\xi^{(1)}$ and $\xi^{(2)}$ is $h(x, y)$ (see (6)); $\eta^{(1)}$ and $\eta^{(2)}$ are independent of each other and of the variables $\xi^{(1)}$ and $\xi^{(2)}$.

Table IV

Numerical results obtained on the basis of Model 3

Population	N	μ^*	σ^{*2}	σ^*	Δ^{*2}	Δ^*	r^*	R^*
Children	244	4.2842	0.1248	0.353	372.93	19.31	—	—
MZ/A	58	4.4233	0.2049	0.453	374.81	19.36	—	—
MZ/B	58	4.3806	0.2082	0.456	452.29	21.27	—	—
MZ pairs	58	4.4021	0.2067	0.455	415.96	20.40	0.4857	0.4590
DZ/A	41	4.5560	0.1470	0.383	1296.80	36.01	—	—
DZ/B	41	4.3508	0.2577	0.508	475.53	21.81	—	—
DZ pairs	41	4.4556	0.2042	0.452	859.57	29.32	0.6659	0.6430

and their density function is $g(z)$ (see (18)); BU values, belonging to different twin pairs are also considered to be independent.

In this case the method of evaluation of the estimates μ^*, σ^{*2} and Δ^{*2} is the same as above (i.e. it consists of solving the equation system (20)), except that the moments are to be determined by considering both members of the twin pairs (see (8)). The estimates r* and R* are evaluated—as in Model 1—according to the formulas (10) and (11), respectively.

Numerical values of the parameters, estimated by applying Model 3, are shown in two Tables: the populations in Table IV are the same as in Tables II and III, while Table V contains results for subpopulations grouped according to sexes.

Table V

Numerical results obtained on the basis of Model 3, related to populations consisting of persons of the same sex

Population	N	μ^*	σ^{*2}	σ^*	Δ^{*2}	Δ^*	r^*	R^*
MZ/A males	27	4.4997	0.1177	0.343	706.69	26.58	—	—
MZ/B males	27	4.3723	0.1759	0.419	384.34	19.60	—	—
MZ pairs males	27	4.4371	0.1482	0.385	545.91	23.36	0.5965	0.5818
MZ/A females	31	4.3637	0.2625	0.512	272.16	16.50	—	—
MZ/B females	31	4.3927	0.2256	0.475	580.57	24.10	—	—
MZ pairs females	31	4.3778	0.2449	0.495	418.47	20.46	0.4479	0.4179
DZ/A males	18	4.6789	0.1019	0.319	1124.28	33.53	—	—
DZ/B males	18	4.3499	0.1830	0.428	409.31	20.23	—	—
DZ pairs males	18	4.5215	0.1489	0.386	804.37	28.36	0.2872	0.2710
DZ/A females	23	4.4445	0.1974	0.444	1226.08	35.02	—	—
DZ/B females	23	4.3659	0.2879	0.537	730.12	27.02	—	—
DZ pairs females	23	4.4035	0.2463	0.496	935.27	30.58	0.8563	0.8407

Model 4

This model is a generalization of Model 3, namely the random variable

$$\zeta = \xi + \eta(\xi)$$

is considered, where for any fixed value $\xi = x$ $(0 < x < +\infty)$, $\eta(x)$ is assumed to be a random variable having a density function

$$g(\alpha, \beta, x; z) = \frac{1}{\sqrt{2\pi(\alpha + \beta x)}} e^{-\frac{z^2}{2(\alpha+\beta x)}} \quad (-\infty < z < +\infty)$$

(i.e. the variance of the "random error" $\eta(\xi)$ is assumed to be a linear function of the "place of observation" ξ)*.

In this model four parameters (μ, σ^2, α, β) are to be estimated (in the bivariate case an additional parameter r is considered). On the analogy of Model 3, in this case four empirical moments must be evaluated and the solution of the equation system obtained leads to that of a seventh-order algebraic equation for the variable Q. The details will not be discussed here.

The numerical results are listed in Table VI.

Table VI

Numerical results obtained on the basis of Model 4 $(\Delta^* = (\alpha^* + \beta^* m_1)^{1/2})$

Population	N	μ^*	σ^{*2}	σ^*	α^*	β^*	Δ_0^*	r^*	R^*
Children	244	4.2852	0.1227	0.350	355.76	0.4155	19.69	—	—
MZ/A	58	4.4311	0.1892	0.435	158.91	4.1067	23.20	—	—
MZ/B	58	4.3899	0.1898	0.436	214.78	4.6791	25.09	—	—
MZ pairs	58	4.4106	0.1897	0.436	190.26	4.3701	24.20	0.5279	0.5045
DZ/A	41	4.5661	0.1266	0.356	971.40	5.5608	39.26	—	—
DZ/B	41	4.4213	0.2265	0.476	2.06	9.0358	28.28	—	—
DZ pairs	41	4.4687	0.1780	0.422	459.68	7.2092	33.87	0.7638	0.7474

Comparative tests of the models. Analysis of the numerical results

In the case of Model 1 and Model 2 the goodness of fit of the estimated distributions to the empirical data was tested. For this purpose the χ^2-test was applied: the domain of measurement was divided into five parts being

* This assumption was suggested by an earlier experience, namely when the individual variability of 16 kindergarten children was tested. The intraindividual variance of the BU values seemed to depend on the average value belonging to the test person (see FORRAI et al. [8], [9]).

equally probable in respect of some hypothetical distribution (one of the estimated distributions); consequently, according to the null hypothesis, each part was expected to contain about 20% of the empirical data. The dividing points were given by the corresponding fractiles of the distribution, i.e. by those values x_p for which the equations

$$\Phi\left(\frac{\ln x_p - \mu^*}{\sigma^*}\right) = p \tag{21}$$

and

$$\Phi\left(\frac{\ln (x_p - \tau^*) - \mu^*}{\sigma^*}\right) = p \tag{22}$$

hold for Model 1 and Model 2, respectively, with $p = 0.2$, 0.4, 0.6 and 0.8 ($\Phi(x)$ denotes the distribution function of the standard normal distribution). The solutions of the equations (21) and (22) are given by

$$x_p = e^{\sigma^* \Phi^{-1}(p) + \mu^*}$$

and

$$x_p = e^{\sigma^* \Phi^{-1}(p) + \mu^*} + \tau^*,$$

respectively. The χ^2-values obtained (with 4 degrees of freedom) are contained in Table VII. In the five populations considered no significant differences could be observed. Comparing the two models, the results for Model 2 showed smaller fluctuations.

In the case of Model 3, evaluation of the fractiles of the distribution functions would have required a great amount of computations; instead of these calculations, a *simulation experiment* was performed concerning the population MZ/A. An "artificial sample" of BU values belonging to 100 "fictive test persons" was taken by generating random numbers with the prescribed distribu-

Table VII

χ^2-values for the test of fit of Model 1 and Model 2
($\mathbf{P}(\chi_4^2 > 7.78) = 0.1$)

Population	N	Model 1	Model 2
Children	244	5.45	1.25
MZ/A	58	1.17	1.31
MZ/B	58	0.28	3.21
DZ/A	41	7.17	3.02
DZ/B	41	1.07	4.24

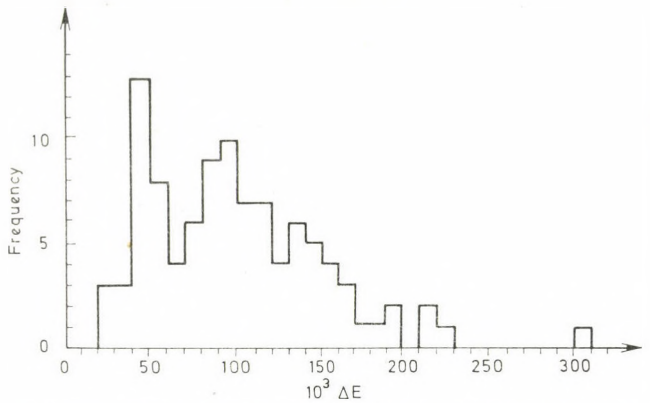

Fig. 2a. Simulated frequency distribution of BU values of 100 "fictive persons". (The artificial sample was taken according to Model 3, i.e. $\zeta = \xi$ (lognormal) $+ \eta$ (normal), with parameters $\mu = 4.423$, $\sigma = 0.4527$, $\Delta = 19.36$, $\mathbf{E}(\eta) = 0$)

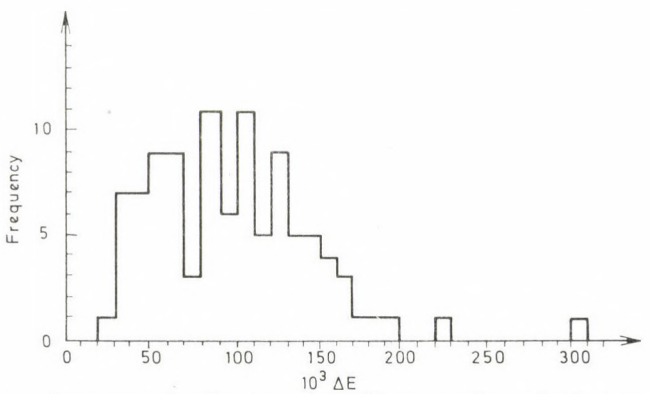

Fig. 2b. Simulated frequency distribution of the "lognormal part" (ξ) indicated in *Fig. 2a*

tion.* The frequency distribution of the generated random quantities is shown in Fig. 2a, while Fig. 2b indicates the frequency distribution corresponding only to the "filtered part" ξ of the random variable $\zeta = \xi + \eta$.

The frequency distribution illustrated by Figure 2a may be considered an interesting contribution to testing the problem of unimodality of probability distributions. If the frequency distribution indicated by the Figure had been obtained from real observations, the theoretical distribution might be supposed to be trimodal. This artificial sample is, however, known to have been generated from a unimodal distribution. Consequently, on the basis of a relatively small sample size it can be rather vaguely judged if the near local peaks of the frequency distribution originated from multimodality of the theoretical distribution or they could be regarded as the results of random fluctuations.

For the standard deviations of the estimated parameters of the two-parametric lognormal distribution the approximation formulas

$$\mathbf{D}(\mu^*) \approx (N-2)^{-\frac{1}{2}}\,\sigma^*\,,$$
$$\mathbf{D}(\sigma^{*2}) \approx 2^{\frac{1}{2}}(N-2)^{-\frac{1}{2}}\,\sigma^{*2}$$

* For simulation methods see e.g. the book of HAMMERSLEY and HANDSCOMB [14].

can be given (in the bivariate case $(N-2)$ must be substituted by $(2N-3)$). For example, by considering the data of Table II, the values $\mathbf{D}(\mu^*) \approx 0.0655$, $\mathbf{D}(\sigma^{*2}) \approx 0.0453$ are belonging to the population MZ/A.

Since it follows from formula (2) that the parameters of the two-parametric lognormal distribution can be estimated on the basis of any two moments of the distribution, the goodness of fit was tested in another way too: namely, the third and fourth empirical moments were also calculated and the parameters were estimated by applying different combinations of the moments. From these investigations the following conclusions could be drawn:

1. The estimates of the parameters, obtained by considering various combinations of moments, did not differ significantly: this fact indicated that the two-parametric lognormal distribution fitted well to the empirical data at the first approach.

2. The estimated values of the parameters did not fluctuate randomly but μ^* showed an increasing tendency while σ^{*2} showed a decreasing one for each population tested, when the estimates were based on higher order moments. This fact suggested that two parameters might be not enough to give an exact characterization of the distribution of the BU values.

Comparing the data of Tables II and III, it can be said that the corresponding (μ^*, σ^{*2}) values are rather different in the case of Model 1 and Model 2, and the differences are to be considered statistically significant or nearly significant.

The differences between the data of Tables II and IV seem to be much smaller. Nevertheless, the differences in respect of the Children population is remarkable, considering that in this case the value of N is relatively large and thus the parameters can be estimated more precisely ($\mathbf{D}(\mu^*) \approx 0.0272$, $\mathbf{D}(\sigma^{*2}) \approx \approx 0.0163$). Although Model 3 has not been fully verified by statistical analysis, it seems to have a real background; namely the existence of a measurement error can be assumed and the results obtained for the value of Δ (20—30 units) have come up to the preliminary expectations.

In spite of the differences of the estimated values of the parameters, the curves of the various density functions were rather near to each other (see Fig. 3*).

Model 3 was applied to investigate populations consisting of males and females separately. The results contained in Table V showed that the σ^{*2} values of the distributions fitted to populations consisting of males were, although not significantly, smaller i.e. the distributions of the BU values were slightly more concentrated for males than for females. The average BU values were, however, almost the same for both sexes. Therefore, even if the distribu-

* As the random variable ζ defined in Model 3 is concerned, only its "filtered part" (i.e. the lognormal distribution of ξ) was illustrated, the density function of ζ would be slightly flattened, owing to the "smoothing effect" of η.

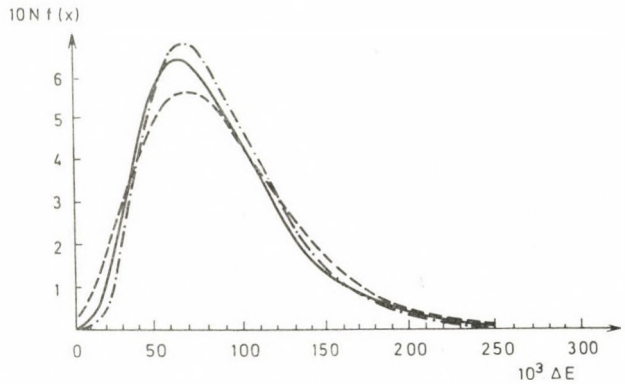

Fig. 3. Estimated frequency curves for several fitted distributions in the case of population MZ/A (cf. Table I). The curves indicate the values of $y = 10\,Nf(x)$, where $f(x)$ is the corresponding estimated lognormal density function ($N = 58$): ——— Model 1, $\mu^* = 4.4058$, $\sigma^{*2} = 0.2399$; — — — Model 2, $\mu^* = 4.7858$, $\sigma^{*2} = 0.1322$, $\tau^* = -35.62$; — · — · Model 3, (only the lognormal part), $\mu^* = 4.4233$, $\sigma^{*2} = 0.2049$

tion of the BU values for a mixed population could be characterized theoretically by a bimodal distribution, the bimodality would hardly originate from the inhomogeneity of the sample in respect to sex.

The results obtained by applying Model 4 have some discouraging features; namely

1. the values of σ^{*2} differ only slightly from the corresponding ones of Model 3,

2. the values of β^* are quite different in children than in adults,

3. the values of α^* are strongly fluctuating.

Despite these facts, the estimated β^* values must refer to the existence of a slightly increasing tendency in the variance of the observations (an exact statistical test for significance does not seem to be easily constructed).

The intra-pair correlation of the BU values proved to be positive both in MZ and DZ twins and for all the models considered.* The connection between the members of twin pairs was not found to be closer for the MZ twins (even the estimated values were in some cases smaller in MZ twins than in DZ twins).

Differences found between the MZ and DZ characters in respect to the distributions of BU values belonging to the populations consisting of the members of MZ and DZ twin pairs are not greater than between the subpopulations DZ/A and DZ/B.

In addition to the comparisons described above, several other factors which might eventually have some influence on the phenomenon of BU were also examined.

1. Is there a closer connection between the BU values of twin pairs living together than of those living apart? A negative answer was obtained.

2. Does age influence the BU values or the intra-pair deviations of BU values? The results obtained for MZ and DZ pairs were rather different. Owing to the small number of sample elements no definite tendency could be stated.

* A t-test was used for the value of r^*.

3. Does the preference or refusal of beetroot have an influence on the BU value of a person? As the great majority of the persons were fond of beetroot, no statistical analysis could be performed.

Discussion

The earlier studies of BU were generally based on visual evaluation of the urine. This technique is only capable to form qualitative categories i.e. the test persons can be grouped into a few (mostly two) classes.

Obviously, the fact that instrumental measurements give exact numerical values instead of a qualitative classification, can be regarded as a great advance in the investigation of the phenomenon. Furthermore, they make the examinations more objective, the arbitrariness of the *a priori* determination of the qualitative categories can be eliminated, and increase the exactitude of the measurements. The quantitative data allow a probabilistic treatment of the phenomenon.

The present twin studies have been performed in order to furnish new data on the hereditary character of the BU. On the basis of the results the following facts weaken the hypothesis of genetic origin (or at least the Mendelian two-allele mode of inheritance).

1. It can be expected from the theory of twin studies that the intra-pair concordance of the MZ pairs is much higher for a hereditary character than that of the DZ pairs; our numerical results did not satisfy this condition.

2. The frequency distribution of the BU values can well be approximated by distributions having unimodal density functions. According to the generally prevailing view, unimodality is indicating that no simple Mendelian mode of inheritance can be retained, but a strong environmental influence or the role of more genes (or both) must be supposed.

The influence of genetic factors in the intensity of BU, however, cannot be excluded, as

1. the intra-pair correlation differs significantly from zero both in the MZ and the DZ pairs;

2. the bimodality cannot completely be excluded although such an obvious multimodality of the distribution of the BU values cannot be expected as it was found e.g. in the cases of the PTC taste sensitivity and the acetone smelling ability (see [4], [5]);

3. there was no remarkable difference in the distribution of the BU values of twins living together and of those living apart; this is not in accordance with the effects of the environment.

Our investigations have thus made more conceivable the conflicting standpoints in the literature referring to the existence of a genetic background of BU. In addition to the methodological weaknesses mentioned, many authors have based their opinion on a small sample size that sharply reduced the reliability of their findings.

Thus, on the basis of the results, the hereditary character of the intenisty of betaninuria cannot sufficiently be verified, and to answer reliably the question of inheritance, BU studies on much greater population samples are needed.

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CYTOCHEMICAL DETECTION OF NICKEL IN THE MYOCARDIUM AFTER ACUTE CARBON MONOXIDE INTOXICATION

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The dimethylglyoxim cytochemical method was used for detecting endogenous nickel (Ni) in the canine and human myocardium. Electrondense deposits were observed in the myocardial cells and the wall and lumen of capillaries of the dog heart when after CO-intoxication the blood COH*b* level exceeded 30%. Energy-dispersive microanalysis proved the presence of Ni in the reaction product.

Detection of the Ni-reaction product in the myocardium of human cadavers may be of forensic importance, since the reaction is resistant to post mortem autolysis and may help to identify the cause of death.

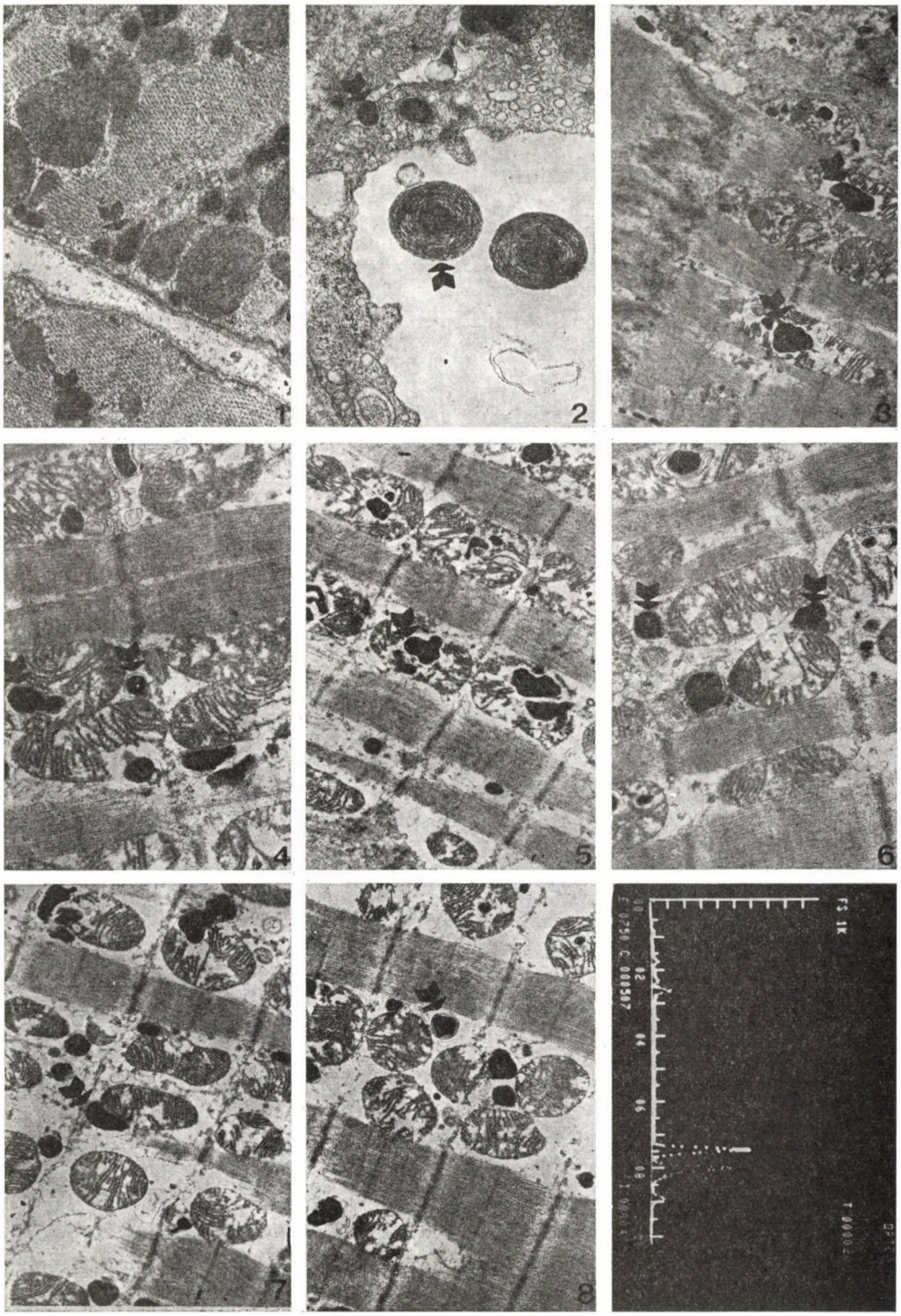
A cytochemical method has been worked out for the visualization of subcellular nickel distribution in the isolated rat heart [2, 6]. The hypoxic-toxic ultrastructural alterations following carbon monoxide poisoning [1, 7] were mitigated by simultaneous perfusion of nickel chloride [4]. The study was designed to analyse the subcellular localization of nickel in the dog heart after acute CO-inhalation or in the myocardium of CO intoxicated human cadavers. The latter analysis was carried out to investigate the effect of post mortem autolysis on the stability of the nickel-dimethylglyoxim complex.

Materials and Methods

Carbon monoxide inhalation was performed in 16 pentobarbital-anaesthetized open chest dogs. Tissue samples were excised from the left myocardium at different times after CO-inhalation with parallel spectrophotometric estimation of the blood carboxyhaemoglobin (COHb) levels. The samples were fixed in 2.5% glutaraldehyde and then incubated in 0.1% dimethylglyoxim (Reanal, Budapest) soluted in 70% ethanol for 5 minutes. One per cent OsO_4 was used for postfixation. The human myocardial specimens were treated similarly. Ultrastructural examination was performed by a JEOL 100B electronmicroscope and the chemical composition of the reaction product was analysed by an energy-dispersive spectrometer (ORTEC) [6].

Results

Characteristic nickel-dimethylglyoxim electrondense deposits were detected in the canine myocardial cells (Fig. 1) and in the lumen and wall of capillaries (Fig. 2) when after an acute CO-intoxication the blood COHb level exceeded 30 relative percents. At higher COHb concentrations the reaction product was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present in the mitochondria as well (Fig. 3). Intracellular oedema characteristic of CO-intoxication could also be observed (Figs 4–6) and the number of Ni--particles was high in the oedematous regions.

Nickel-dimethylglyoxim particles were present in the human myocardial cells (Figs 7 and 8).

The energy-spectrum obtained by energy-dispersive microanalysis (Fig. 9) showed a distinct peak at 7.5 keV (specific Ni-peak) and several other peaks which could be attributed to the technique used; these were copper (Cu) peaks due to the use of copper grids and a dimethylglyoxim (D) peak due to its presence in the material.

Discussion

The present experiments showed that besides the localization of exogenous Ni, the dimethylglyoxim cytochemical method is a good tool for the detection of endogenous Ni in the myocardium after CO-inhalation (acute dog experiments) or of the endogenous Ni-accumulation and/or release after post mortem autolysis (human cadavers). Under normoxic conditions, no endogenous Ni could be detected in the myocardium.

After acute CO-inhalation, Ni-deposits could only be detected in the lumen of capillaries of the severely hypoxic heart muscle if the blood COHb level exceeded 30%.

The fact that post mortem autolysis did not influence the detectability of the Ni-dimethylglyoxim complex is of particular importance in forensic medicine since a positive reaction may indicate a CO-intoxication as a possible cause of death.

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Figs 1—6. Electroncytochemical detection of Ni-dimethylglyoxim deposits in the myocardial cells and capillaries of the dog heart exposed to CO-intoxication
Fig. 7—8. Ni-dimethylglyoxim deposits in human myocardium (post mortem analysis)
Fig. 9. Energy spectrum of the reaction product obtained by energy-dispersive microanalysis (Ni-peak: 7.5 keV; Cu: copper; D: dimethylglyoxim)

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EFFECT OF VERAPAMIL AND PHENOXYBENZAMINE ON NICKEL-INDUCED CORONARY VASOCONSTRICTION IN THE ANAESTHETIZED DOG

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(Received October 17, 1981)

Ni-induced coronary vasoconstriction has been studied in the dog heart in situ in the presence of the selective Ca-antagonsit verapamil, and after blocking the alpha-receptors with phenoxybenzamine (PBZ).

Verapamil totally abolished the coronary blood flow (CBF) and basal conductance (BC) decreasing effect of low doses of Ni^{2+} (0.02–0.2 mg/kg^{-1}). The effect of higher doses of Ni^{2+} (2.0–20.0 mg/kg^{-1}) was reversed by verapamil, i.e. high doses of exogenous $NiCl_2$ increased CBF and BC in the presence of verapamil. The CBF decreasing effect of nickel was not significantly influenced by PBZ pretreatment.

The results indicate that trace amounts of exogenous $NiCl_2$ induce coronary vasoconstriction in the dog heart in situ by enhancing Ca^{2+}-influx into vascular smooth muscle cells, which is not mediated by alpha-receptors.

Studies on isolated rat hearts [4] and canine coronary artery strips [5] showed that nickel ions at low concentration (10^{-8}–10^{-7} M) induce vasoconstriction by enhancing Ca^{2+}-influx into coronary vascular smooth muscle cells. Correlation of haemodynamic and coronary blood flow changes after i. v. injection of various doses of exogenous $NiCl_2$ in anaesthetized dogs showed that the reduction of coronary blood flow (CBF) by trace amounts of nickel may be regarded as a local action of this trace element on coronary vessels [2, 3].

The role of Ca^{2+} in Ni-induced coronary vasoconstriction in the dog heart in situ is still unknown. VAN BREEMEN et al. [6] found that stimulation of coronary alpha-receptors enhances Ca^{2+} influx into vascular smooth muscle cells. The possibility thus exists for the Ni^{2+} action of being mediated by alpha-receptors in the coronary vessels. In order to gain more information about the possible physiological and/or pathological significance of nickel ions, it was mandatory to study the possible action mechanism of Ni-induced coronary vasoconstriction in the open chest dog heart in situ in the presence of the selective Ca antagonist verapamil, and in the case when the alpha-receptors were blocked by phenoxybenzamine (PBZ).

Materials and Methods

Mongrel dogs of either sex weighing 16 to 33 kg were anaesthetized by glucochloralose (100 mg/kg^{-1} body weight) with additional anaesthetic given as needed to maintain a constant level of anaesthesia. The animals were immobilized by flaxedyl (2 mg · kg^{-1}) and pulmonary ventilation was maintained by a positive pressure respirator (Harvard) with room air enriched

by 100% oxygen. Blood gases and pH were monitored (Radiometer Copenhagen Type ABL 1) and kept within acceptable ranges. A femoral artery and vein were cannulated for continuous monitoring of mean arterial blood pressure (MABP) and for intravenous administration of fluids and drugs. The heart was approached through a midsternal incision. An electromagnetic flow probe (Statham Sp 2022) was placed around the ascending aorta to measure cardiac output (CO), and around the left anterior descending (LAD) coronary artery for monitoring coronary blood flow (CBF). Conventional (lead LI) and epicardial ECG were monitored and spontaneous heart rate (HR) was continuously recorded by an integrator fed by the R-wave signal of ECG. A stainless steel needle was inserted into the left ventricle and dp/dt was continuously recorded by a derivative circuit fed by the ventricular pressure signal. Recording of all parameters was carried out on a 12-channel Grass polygraph (Model 7PCM 12). Basal coronary conductance (BC) was calculated.

Haemodynamic, cardiac and coronary flow data were obtained in 7 animals 30 minutes after the start of recording when all parameters had reached a stable level. Verapamil (Verpamil®; Orion) was given as a bolus injection (0.2 $mgkg^{-1}$) intravenously over a period of 2 to 3 min. This was followed by a constant infusion of the drug using a Harvard pump set to deliver 0.01 $mgkg^{-1}min^{-1}$. After the effect of verapamil infusion had become stable (30 min) increasing doses of $NiCl_2$ (0.02; 0.2; 2.0; 20.0 mg/kg^{-1}) were administrated intravenously at 20 min intervals in a cumulative manner. In the second series ($n = 7$) of the experiments, phenoxybenzamine (PBZ) (Smith, Kline and French) (1 $mgkg^{-1}$) was administered i.v. 18 hours before beginning the experiments.

The mean and standard error of the mean ($\bar{x} \pm SEM$) of grouped experimental data was calculated. The statistical difference between means was estimated by the paired and unpaired Student's t tests.

Results

The effect of various doses of Ni^{2+} on cardiac output (CO), mean arterial blood pressure (MABP), left ventricular contractility (dp/dt max) and heart rate (HR) in the absence and presence of verapamil or PBZ are summarized in Fig. 1. Low doses of exogenous $NiCl_2$ (0.02—0.2 mg/kg^{-1}) did not change any of the haemodynamic or cardiac parameters studied. The presence of verapamil was ineffective and PBZ pretreatment potentiated the Ni action in the case of MABP only: $NiCl_2$ caused a significant reduction of MABP in the presence of PBZ. Higher doses of $NiCl_2$ (2.0—20.0 mg/kg^{-1}) caused a decrease of CO, MABP and dp/dt max which was not influenced by either verapamil or PBZ. In the presence of verapamil the highest $NiCl_2$ dose caused a significant reduction of HR.

In sharp contrast, the effect of various doses of Ni^{2+} on coronary blood flow (CBF) and basal coronary conductance (BC) was significantly altered in the presence of verapamil or when the alpha-receptors had been blocked by PBZ (Fig. 2). Verapamil totally abolished the coronary blood flow and basal conductance decreasing effect of lower doses of Ni^{2+} (0.02—0.2 mg/kg^{-1}). The effects of higher doses of Ni^{2+} (2.0–20.0 mg/kg^{-1}) were not only prevented but reversed by verapamil, i.e. high doses of exogenous $NiCl_2$ increased CBF and BC in the presence of the selective Ca-antagonist. The CBF decreasing effect of nickel was not significantly influenced by PBZ pretreatment. The BC decrease by the lower Ni^{2+} doses was, however, totally abolished, but the effect of higher Ni doses was not significantly altered by the drug.

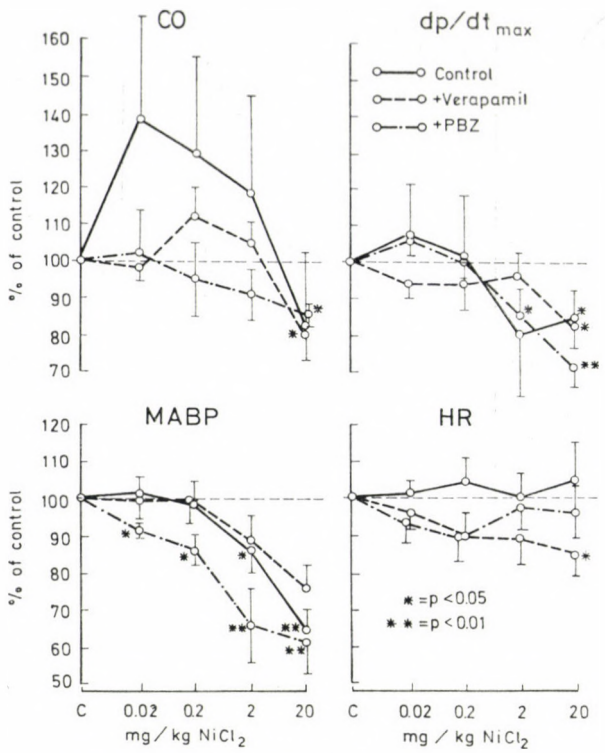

Fig. 1. Effect of increasing doses of $NiCl_2$ (0.02–20.0 $mgkg^{-1}$) on cardiac output (CO) mean arterial blood pressure (MABP), left ventricular contractility (dp/dt max) and spontaneous heart rate (HR) in the absence (control) and in the presence of verapamil or PBZ ($\bar{x} \pm$ S. E.). Asterisks represent statistically significant differences from control values

Discussion

The present results showed that a constant verapamil infusion totally abolished the coronary vasoconstriction induced by low Ni^{2+} doses (0.02 and 0.2 mg/kg^{-1}). These data clearly indicate that trace amounts of exogenous $NiCl_2$ induce coronary vasoconstriction by enhancing Ca-influx into vascular smooth muscle cells of the dog heart in situ as also observed in the isolated rat heart [4] and isolated canine coronary artery [5]. The fact that verapamil not only prevented but also reversed the coronary action of higher Ni doses suggests that $NiCl_2$ induces an increase of coronary conductance if the dominating vasoconstrictor action is eliminated. The exact nature of this dual action of Ni^{2+} on the coronary vessels of the dog heart in situ needs further investigations. The finding that PBZ caused only partial inhibition of Ni-induced coronary vasoconstriction has confirmed earlier results concerning the isolated perfused rat heart [1] and indicates that beside stimulation of alpha-receptors the action of Ni on coronary vessels involves other mechanisms as well.

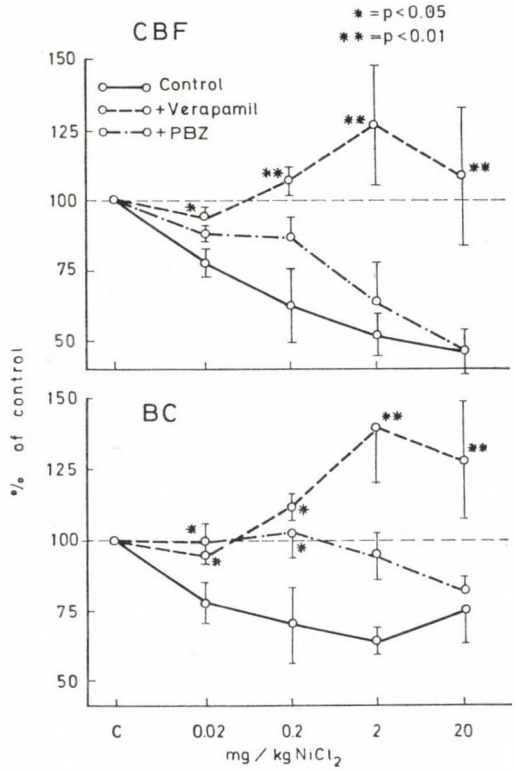

Fig. 2. Effect of increasing doses of $NiCl_2$ (0.02–20.0 $mgkg^{-1}$) on coronary blood flow (CBF) and basal coronary conductance (BC) in the absence (control) and in the presence of verapamil or PBZ ($\bar{x} \pm$ S. E.). Asterisks represent statistically significant difference from control values

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Recensiones

A. G. Brown and M. Réthelyi (Eds)

Spinal Cord Sensation, Sensory processing in the Dorsal Horn

Scottish Academic Press, Edinburgh 1981. 341 pages, Price L 15.00

During the past two decades a significant amount of new information has accumulated about the structure and function of the dorsal horn of the spinal cord. The exciting advances in this field are summarized in this book, the proceedings of a Satellite Symposium of the 28th International Congress of Physiological Sciences, Budapest 1980.

Most of the papers are concerned with the morphology of the dorsal horn and pharmacology of synaptic transmission in this part of the spinal cord. Among others geometry, significance of laminar arrangements, synaptic organization, plasticity, primary afferent input, projection patterns of peripheral sensory nerves, somatotopic organization of cutaneous projections, spinal terminations of subcutaneous high-threshold mechanoreceptors, substantia gelatinosa interneurones, ultrastructure of functionally identified neurones, dendritic trees of dorsal horn cells, Lissauer's tract ascending pathways from the dorsal horn, collateral branching of ascending and descending spinal tracts, peptidergic mechanisms, serotonin/enkephalin interactions, effect of GABA, reactions of the substantia gelatinosa to injury of peripheral sensory axons and alterations after deafferentation are discussed. In addition, brief reports of Working Parties of the Symposium on terminology, transmitters in the dorsal horn and plasticity are included.

The book is an important source of up-to-date information about research being done on the dorsal horn. It should be of interest to all researchers working in neuroscience, i.e. to neuroanatomists, neurophysiologists, neurochemists, neuropharmacologists. But it can be recommended also to clinicians, primarily to neurologists and to all those who wish to learn about the recent advances in knowledge on the dorsal horn of the spinal cord.

B. Halász

J. Hideg and O. Gazenko (Eds)

Gravitational Physiology

Advances in Physiological Sciences, Vol. 19. Pergamon Press, Oxford and Akadémiai Kiadó, Budapest 1981. 315 pages

On the initiative of the International Union of Physiological Sciences (IUPS) the Second Gravitational Symposium took place at the 28th IUPS Congress organized in Budapest in July, 1980.

On September 25, 1979, aboard an unmanned spacecraft (COSMOS 1129) launched by the Soviet Union a variety of biological experiments were carried out. The primary task was to investigate how organisms adapt to hypogravity and then readapt to the gravity of the Earth following spaceflight. Most of the papers presented in this volume deal with the results of the above joint USSR/USA space flight experiments.

BJURSTEDT, the first chairman of the Commission of Gravitational Physiology in an introductory lecture gave a brief summary of the development of gravitational physiology and the adaptive reactions developing in physiological systems in response to space flight.

Besides the problems of adaptation to changed gravity many lectures deal with the functional, biochemical and morphological changes of gravity-dependent systems: the vestibular system, bone, skeletal muscle and mineral metabolism.

Mention has also been made of new models, mainly hypokinesis and orthostasis, which seem to be adequate methods for simulating the changes elicited by long-term space flights.

New measures have been described about the minimum value of centripetal forces required to maintain good health conditions aboard space crafts during long term flights.

This volume may attract attention of both physiologists and biochemists dealing with adaptational problems of living organisms.

A. G. B. KOVÁCH

Thaddeus MANN and Cecilia LUTWAK-MANN

Male Reproductive Function and Semen

Springer Verlag, Berlin, Heidelberg, New York 1981.

The authors' activity is well known in the scientific world of their special line. Since the publication of their work "The Biochemistry of Semen and of the Male Reproductive Tract" in 1964, their field of research has been extended. Recent information carefully harmonized with former knowledge can be found in their new book of nearly 500 pages, containing 10 chapters, nearly 3500 references and a detailed and well arranged index.

Chapter I is a guide of general character concerning the physiological basis of seminal power and composition of the semen.

Chapters II and III deal with methodological problems.

Chapters IV to VII discuss the endocrine and exocrine function of the testis, the function of the epididymis and of the ductus deferens, and investigate the role of spermary, prostate and accessory sexual glands in the formation of the final composition of the ejaculate.

Chapters VIII and IX deals with the biochemistry of semen.

Chapter X discusses the positive and negative pharmacological factors influencing fertility, and the role of further factors.

The book investigates in a particularly detailed manner the question of seminal power, and beyond the human points of view, it analyses the individual problems in a general biological sense. The studies performed in different species are means to estimate the questions of human seminal power from a wider aspect.

The author's intention has been to provide a conscise handbook. They discuss the latest results and also the scientific progress of the field of research with great historical fidelity, also presenting the latest scientific trends. This work of encyclopaedic character will become a fundamental source for andrologists as well as zoologists. The book is a must for the library of all experts of the subject.

F. BALOGH and G. CORRADI

F. OBÁL and G. BENEDEK (Eds.)

Environmental Physiology

Advances in Physiological Sciences, Vol. 18. Pergamon Press Oxford and Akadémiai Kiadó, Budapest 1981. 371 pages

Environmental physiology comprises an extremely wide field of research of applied physiology, and it is almost impossible to summarize every problem in a single section of a congress, be it as big as the 28th IUPS Congress was in Budapest 1981. The most current problems of environmental physiology have been summarized in this volume.

J. ASCHOFF, one of the invited lecturers, spoke about the regulatory mechanisms of circadian system properties. He discussed the role and multiplicity of both the central and the self-sustaining oscillators at the periphery.

Another invited lecture held by R. HELLON analysed the recent advances of new techniques in studying thermoregulation and emphasized the role of thermosensitive systems outside the CNS in the overall thermoregulation of the organism.

Other main subsymposia were: "Osmoregulation" in which emphasis was laid upon outlining the role of neural networks; "Regulation of the sleep-waking system" where the mechanism of action of the most important neurogenic substances was discussed; "Sport physiology", where attention was directed toward the problems of hormonal (hypothalamic, pituitary and adrenal systems and anabolic preparations and pharmacological regulatory mechanisms, with special emphasis on beta blocking agents). Other symposia of interest were held under the titles "Mammalian nervous system under pressure" and "Physiology of static effert".

This volume will attract the attention of physiologists working in the field of applied physiology and of related sciences.

A. G. B. KOVÁCH

SCHUBERT, E.

Grundriss der Physiologie des Menschen

Verlag Volk und Gesundheit, Berlin, 1981. 176 pages, 70 figures, 17 tables. Price 12,90 M

The introduction of physiology in the GDR medical highschool programme as a separate subject necessitated the publication of this book. The author is the head of the Physiological Department of Humboldt University, Berlin.

The textbook includes the functional views on healthy living and the learning of functional diseases. The text is based upon the knowledge of the student after the 8th grade. It states facts without explaining them in detail but does convey their application in practice.

In the text only the SI system is used and it is explained how to convert the old units into those of the SI-system.

First subjects to be dealt with are surveyed followed by a chapter on energy balance and metabolism. This chapter discusses respiration, digestion and absorption, haematological functions and also intermediary metabolism.

The chapter "Exchange of information" includes the function of excitable tissue, the physiology of sense organs and the central nervous system.

The mechanisms regulating homeostasis, energy metabolism, endocrine functions and acclimatization are summarized in a chapter entitled "Physiological regulation".

The last chapter considers physiological changes from the embryo stage up to old age.

The textbook is a concise summary of physiology. The quality of the text makes it recommendable for highschool and college students of health.

Éva Szőcs

Colin J. SCHWARTZ, Nicholas T. WERTHESSEN and Stewart WOLF (Eds)

Structure and Function of the Circulation

Vol. 3. Plenum Press, New York and London 1981. 551 pages with 148 figures

A comprehensive series of books on the structure and function of the circulation of blood and lymph evolved basically from the scientific sessions of three interdisciplinary international conferences held in Lindau in 1970, in Heidelberg in 1973, and in Totts Gap, Pennsylvania in 1976. This third volume of the series represents a collection of essays covering a broad area of vascular biology. The topics included are arterial histochemistry, proteoglycans of the vessel wall, cell membrane surface structure, integrity, receptor properties and contractile proteins of vascular endothelium, as well as rigorous mathematical models of protein transport. Other important contributions to the biology of arterial smooth muscle included into the volume cover differentation and phenotypic modulation, contraction and innervation of muscle cells in culture and the electrophysiology of muscle contraction.

The carefully selected essays written by outstanding experts from around the world will serve as a useful source of references for students and researchers of vascular biology and diseases.

E. MONOS

H. G. SCHWEIGER (Ed.)

International Cell Biology 1980—1981

Springer Verlag, Berlin—Heidelberg—New York 1980. 1033 pages, 595 figures. Price: approx. US $ 61.00

This large book, which on its cover states proudly that "this book will remain a useful source of information to biologists, medical researchers and biochemists for years to come", contains the scientific material of the lectures presented at the Second International Congress of Cell Biology in West-Berlin in September, 1980. The statement on the cover will prove right. There are several papers in the volume which may begin new fields in research, there are new names introduced which are not yet generally accepted but will soon become well known. The volume does not cover the whole field of cell biology, but its content is very thought-provoking and provides much new information which otherwise could be collected only with the assistance of a whole literature monitoring staff.

The contents are divided into six chapters, which are as follows: genome and gene-expression, cytoskeleton, cytopathology and pathogenesis, differentiation and development, membrane and cell surface, and functional organization.

It would be very difficult to discuss only one of it (or one or two out of the 111 lectures) in the framework of this review. It should, however, be stated that the majority of the authors has presented new experimental data based on well-designed and proper experiments. Some of the papers give a survey of the literature in a concise form.

As the extent of the volume indicates, the material presented at the congress was very large and the participants could not listen to all of the papers presented. So we may say that it is very important that this book should reach all researchers working in the field of biology. This is indispensable in our era when the development of cell biology is extremely rapid, publications protracted, and the places of publication are very scattered.

The figures in the volume are demonstrative and of good quality. The text of the lectures is followed by specific reference lists. There is an index in the book, which makes orientation easy.

Gy. CSABA

Lutz VOLRATH

The Pineal Organ

Handbuch der mikroskopischen Anatomie des Menschen, Volume VI/7

This new volume of the series has been devoted to the memory of Professor Wolfgang BARGMANN and Professor Sir Francis KNOWLES. It is a comprehensive survey of the research concerning the pineal gland.

The book is introduced by highlighting two important events of pineal gland research. The first was the discovery that this organ produces a hormone-like indolamine substance termed melatonin. The second and even more important event was the recognition of the fact that unlike other endocrine glands the pineal organ is under the direct influence of environmental factors among which photic stimuli appear to play a leading role. In the author's opinion, interpretation of the pineal gland as a neuroendocrine transducer similar to the anterior and posterior pituitary and adrenal medulla is acceptable.

All the problems connected with the pineal organ are discussed functionally and a number of comparative phylogenetic data are also presented. There is clear evidence that in lower vertebrates the pineal organ differs from that in higher vertebrates; while in fish, amphibians and reptiles the epiphyseal complex contains photoreceptor cells similar to those in the lateral eyes. It is capable of responding to light directly; but the pineal in birds and mammals is lacking typical photoreceptors and is only indirectly light-sensitive.

It has recently been shown that the mammalian pineal possesses receptors for sex steroids and that these and other hormones secreted by the adrenal medulla during stress influence pineal function. These observations have led to the view that the pineal gland is an endocrine-endocrine transducer.

Although the main logic of the book is the morphology of the pineal gland, biochemical, pharmacological and physiological data have also been incorporated. The mammalian pineal

rgan shows biochemical individuality: the mammalian pinealocytes particularly active in monoamine biosynthesis, resemble neuronal and retinal cells. They have two striking features: the synaptic ribbons and the cytoplasmic processes; the former may relate to photic stimuli, the long cytoplasmic processes have bulbous ends with electron-lucent and dense-core vesicles.

The book considers in five main chapters the pineal organ of mammals, lampreys, fish, amphibians, reptiles and birds. The list of references contains nearly 3000 papers including 44 review articles. It concludes with indexes of authors and subjects. Descriptions are illustrated by 190 photographs and drawings. In particular the transmission and scanning electron microscopic pictures are of high quality. The explanatory schematic diagrams are didactic, easy to understand.

The book is written in a concise, readable style which makes it a useful and interesting work not only for the specialist but for all students of neurohormonal regulation.

Teréz TÖMBÖL

W. WUTTKE and R. HOROWSKI (Eds)

Gonadal Steroids and Brain Function

Springer Verlag, Berlin—Heidelberg—New York 1981. 373 pages, with 136 figures and 10 tables

The book contains 25 papers and 18 abstracts of posters presented at an IPUS-Satellite Symposium in Berlin, July 1981. The papers cover various aspects of interactions between gonadal steroids and brain function. These include feed-back circuits (M. MOTTA et al., K. BAUER et al., R. KNUPPEN, J. MEITES et al., B. T. DONOVAN and B. GLEDHILL, H. BREUER et al.), steroid receptors (P. W. JUNGBLUT et al., B. MCEWEN), neurotransmitters (W. E. STUMP et al., M. SAR and W. E. STUMPF, W. WUTTKE and T. MANSKY, S. M. MCCANN et al.), sexual maturation (F. DÖCKE, G. DÖRNER, F. NEUMANN and W. ELGER) and a detailed analysis of neural control of hypothalamus-hypophyseal-gonadal function (R. A. GORSKI and M. YANASE, L. M. KOW and D. W. PFAFF, M. KAWAKAMI and J. ARITA, M. J. KELLY et al., D. BECKER et al.). In C. H. SAWYER's "Concluding remarks on gonadal steroids and brain function" the latest developments of these subjects are emphasized.

The book will prove a valuable source of information to those interested in reproductive neuroendocrinology, to endocrinologists, gynaecologists, physiologists as well as scientists working in this field.

Marietta VÉRTES

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ACTA PHYSIOLOGICA

ТОМ 59 — ВЫП. 3

РЕЗЮМЕ

ВЛИЯНИЕ ИЗОПРОТЕРЕНОЛА НА ПОЧЕЧНУЮ ДЕЯТЕЛЬНОСТЬ

Т. ЗАХАЙСКИ, Г. ФЕЙЕШ-ТОТ и Я. ФИЛЕП

В экспериментах на бодрствующих собаках, получивших водную нагрузку, мы сравнивали действие, оказываемое изопротеренолом и вазопрессином на деятельность почек.

Под влиянием введения изопротеренола (12 мкг/кг/час) значительно уменьшились минутный диурез, выделение натрия и калия, а также клиренс свободной воды, в то же время практически не изменились: клубочковая фильтрация, количество протекающей через почку крови, а также распределение в корковых слоях протекающей через почечную кору крови.

В ответ на введение вазопрессина (2 ИЕ/кг/час) возник антидиурез, подобный такового после введения изопротеренола, но выделение натрия и калия значительно возросло.

Поскольку изопротеренол и вазопрессин оказывают противоположное действие на выделение ионов натрия и калия, можно сделать вывод, что в изменениях почечной деятельности, вызванных изопротеренолом, стимулирование секреции вазопрессина не может играть исключительной роли.

ПОВЫШЕНИЕ ПОРОГА ТЕРМОРЕГУЛЯЦИОННОЙ ВАЗОДИЛАТАЦИИ У КРЫС ПОСЛЕ ВВЕДЕНИЯ КАПСАИЦИНА

Ф. ОБАЛ МЛ., Ф. БАРИ, Г. БЕНЕДЕК и Ф. ОБАЛ

В экспериментах на крысах мы исследовали расширение сосудов в коже хвоста животных, как у контрольных, так и предварительно получивших капсаицин, при изменении окружающей температуры (38°С, 39°С и 40°С). При 38°С вазодилатация у предварительно получавших капсаицин крыс была меньше выражена, чем у контрольных животных, тогда как при 40°С вазодилятация была примерно такой же степени, но наступала с запозданием. Мы приходим к выводу, что, после введения капсаицина, порог терморегуляционной периферической вазодилатации повышается.

ДЕЙСТВИЕ ГОРМОНОВ ПРОГЕСТЕРОНА, ТЕСТОСТЕРОНА И ЭСТРОГЕНА НА КОНЦЕНТРАЦИЮ ГОРМОНА ЩИТОВИДНОЙ ЖЕЛЕЗЫ И КОРТИКОСТЕРОНА В ПЛАЗМЕ ЯПОНСКОЙ ПЕРЕПЕЛКИ

ПЕТЕШ и ПЕЦЕЛИ

У самок короткодневных (6L — 18D) японских перепелов радиоиммунологическим методом определяли концентрацию в плазме крови трийодтиронина, тироксина и кортикостерона. В отдельных подгруппах первой экспериментальной группы после овариэкто-

мии птицам имплантировали капсулы *Silastic*, содержавшие прогестерон, тестостерон, эстрон и 17β — эстрадиол или все четыре половых стероида сразу. В этих экспериментах прогестерон незначительно повышал концентрацию тироксина в плазме, тогда как тестостерон, эстрон и 17β — эстрадиол снижали уровень плазменного тироксина, по сравнению с овариэктомизированной группой. Одновременное применение четырех половых стероидов снижало уровень как тироксина, так и трийодтиронина. Прогестерон и 17β — эстрадиол значительно, тогда как эстрон на небольшой степени увеличивают концентрацию кортикостерона в плазме. Во второй серии экспериментов, после овариэктомии и произведенной одновременно оперативной тиреоидектомии, производились те же вмешательства. Будучи применены вместе и по отдельности половые стероиды повышали концентрацию кортикостероидов плазмы до уровня у интактных животных. Как показывают результаты проведенных экспериментов, прогестерон повышает концентрацию кортикостерона в плазме крови у японских перепелок отчасти прямым путем, отчасти же путем увеличения концентрации в плазме тироксина, тогда как эстрон и эстрадиол непосредственно повышают уровень плзаменного кортикостерона.

ВЛИЯНИЕ ИНДОМЕТАЦИНА НА ВНУТРИПОЧЕЧНОЕ КРОВООБРАЩЕНИЕ И ВЫДЕЛЕНИЕ НАТРИЯ И ВОДЫ У АНЕСТЕЗИРОВАННЫХ КРЫС В СОСТОЯНИИ НОРМО- И ГИПЕРВОЛЕМИИ

Й. БАРТА и Ч. ХАБЛИ

Мы изучали влияние индометацина (4 мг/кг), ингибирующего синтез простагландинов, на интраренальное кровообращение у наркотизированных пентоталом (50 мг/кг, интраперитонеально) крыс с нормоволемией или экстрацеллюлярной гиперволемией (после внутривенной инфузии 50 мл/кг 0,9% раствора хлористого натрия) методом Сапирштейна с индикаторной аккумулицией ^{86}Rb.

Определение параметров кровообращения производили через 1 час после введения индометацина.

Были получены следующие результаты:

1. В состоянии *нормоволемии* кровоток в почке, в ее корковом и мозговом слоях не изменяется. Не изменяются также значения регионарного сосудистого сопротивления в почке, а также процентное распределение интраренального кровообращения. Умеренно снижается выделение воды, выделение натрия не изменяется.

2. При *экстрацеллюлярной гиперволемии* умеренно возрастает кровоток в корковом слое и уменьшается в мозговом слое почек; процентное распределение кровотока внутри почки смещается в направлении коркового слоя. Корковое сосудистое сопротивление не изменяется, мозговое увеличивается в небольшой степени. В этом состоянии индометацин не оказывал влияния на диурез соли и воды.

Результаты настоящих экспериментов, проведенных на крысах, дают дальнейшие данные относительно того, что эндогенные простагландины не играют здесь такой решающей роли в интраренальном кровообращении, выделении натрия и воды, как у анестезированных собак.

ВЛИЯНИЕ ИММОБИЛИЗАЦИИ ГИПСОВОЙ ПОВЯЗКОЙ НА УРОВЕНЬ МЕТАБОЛИТОВ В СКЕЛЕТНОЙ МЫШЦЕ, СВЯЗАННЫХ С ЭНЕРГЕТИЧЕСКИМ ОБМЕНОМ

И. СИКЛАИ и Й. ГРОФ

В динамике развития инактивационной атрофии скелетной мышцы, развивавшейся при 1—6-недельной иммобилизации конечности наложением гипсовой повязки, мы определяли с помощью методики изотахофореза содержание ATP, ADP, AMP, CrP, NAD^+ и Pi в медленной *(m. soleus)* и гликолитической, быстрой мышце *(m. gastrocnemius)*.

Содержание ATP и CrP в гликолитической мышце было достоверно выше, а содержание NAD^+ в оксидативной мышце было достоверно выше в нормальном состоянии. Энергетическое хозяйство мышц в первую неделю иммобилизации нарушается, но к шестой неделе, при формировании нового состояния равновесия, на более низком энергетическом уровне, мышцы приспосабливаются к лишению движения. Новые энергетически уровни, характеризующиеся содержанием макроэнергетических метаболитов, не обнаруживают достоверную и существенную разницу, специфическую для разных типов мышц.

НОВАЯ ТЕОРИЯ ВОЗНИКНОВЕНИЯ МИОКАРДИАЛЬНОГО ИНФАРКТА

М. СЕНТИВАНИ, Ч. ВЕТЕШИ, А. ПААЛ, М. ПЕНЗЕШ и ДЬ. ЛЕСКОВСКИ

Введение хлорситого калия (1%, 10%, 31,2%) в перикардиальную жидкость или воздействие на сердце наложением на его поверхность пропитанной KCl фильтровальной бумаги вызывает у собаки, морской свинки и крысы появление типичной для инфаркта т. н. *dome*-подобной ЭКГ. Через 2—3 дня после острого опыта развивается инфаркт. Как *dome*-ЭКГ, так и следующий за ней инфаркт можно предотвратить взятой из перикарда жидкостью, а также тремя синтетическими веществами. Те же защитные вещества, которые предотвращают эффект хлористого калия, предотвращают общую гипоксию организма, вызывающую появление *dome*-ЭКГ. Речь идет об особом пространстве, которое начинается порами и, вероятно, через соединительнотканные щели достигает миокардиальные клет клетки.

В своих предыдущих исследованиях мы показали, что при наложении на эпикардий фильтровальной бумаги, пропитанной I^{125}-альбумином, последний проникает в эпикардиальные щели сердца. Интраперикардиальное введение туши, вследствие физической закупорки пор, препятствует возникновению интраперикардиального эффекта хлористого калия вызываемого этим инфаркта. Это пространство иное, нежели пространство, которое можно достигнуть со стороны коронарных сосудов. Некоторые соединения, так, например норадреналин, не оказывают воздействия через это пространство, другие же вещества попадают в него. Поверхностная оксигенизация эпикардия, воздействуя на пространство, предотвращает воздействие хлористого калия и гипоксии.

ДЕЙСТВИЕ ДЕЗОКСИКОРТИКОСТЕРОНА НА ИНТЕРСТИЦИАЛЬНОЕ ДАВЛЕНИЕ У НОРМАЛЬНЫХ КРЫС

М. ГИЛАНИ, Ш. ШИМОН и А. ДЬ. Б. КОВАЧ

С помощью методики, разработанной *Guyton*, мы определяли изменени интерстициального давления в соединительной ткани спины у крыс, которые наступали в ответ на однократное интраперитонеальное введение дезоксикортикостерона (ДОК). В изменениях межтканевого давления во времени, независимо от введенной дозы ДОК, можно выделить три периода: латентный период, затем период снижения давления и, наконец, период возвращения давления к его контрольному значению. Величина наибольшего снижения давления и время возвращения его к исходному уровню, однако, являются дозозависимыми. В ответ на введение дезоксикортикостерона в дозах: 0,25; 0,50; 0,75; и 1,00 мг на 100 г веса тела интерстициальное давление снижалось соответственно на: 3,2; 7,1; 9,6 и 10,6 мм рт. ст. Исследуя причину изменения давления под действием дезоксикортикостерона, мы пришли к выводу, что это явление нельзя приписать исключительно только т. н. силам Старлинга, по-видимому, здесь играет роль также потеря внеклеточного калия. Эту гипотезу подтверждает также тот экспериментальный факт, что валиномицин, который специфически ускоряет калиевый транспорт, не оказывает влияния на величину изменений интерстициального давления в ответ на введение дезоксикортекостерона, но в то же время значительно укорачивает время протекания изменений.

БЕТАНУРИЯ: ГЕНЕТИЧЕСКОЕ ЯВЛЕНИЕ?

ДЬ. БАНКЁВИ, ДЬ. ФОРРАИ и Д. ВАГУЙФАЛВИ

На 58 однояйцевых и 41 двуяйцевых одного пола близнецах авторы провели определение в моче пигмента бетанина (они назвали это бетанинурией) после приема свекольного сока, имеющего стандартную концентрацию. С помощью введенного ими полуколичественного метода определили значения бетанурии у всех исследуемых лиц. Исследовали несколько математических моделей, чтобы найти такое распределение вероятности, которое хорошо подходит к опытным данным.

Результаты анализов показывают, что наследуемый характер интенсивности бетанурии нельзя принять с полной надежностью, хотя некоторые признаки указывают на то, что генетические факторы могут влиять на это явление. По мнению авторов, более определенная точка зрения в связи с этим явлением может базироваться только на исследовании популяции с большим числом элементов.

ЦИТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ ИОНОВ НИКЕЛЯ В СЕРДЕЧНОЙ МЫШЦЕ ПРИ ОСТРОМ ОТРАВЛЕНИИ ОКИСЬЮ УГЛЕРОДА

Я. БАЛОГ, Г. РУБАНИ, Г. ПОГАЧА и М. ОБЕРНА

Субцеллюлярную цитохимическую локализацию никеля авторы изучали с помощью метода деметилгликоксима; локализацию никеля определяли в миокарде собак вслед за двыханием окиси углерода (CO), а также в сердечной мышце людей, умерших вследствие отравления окисью углерода. Показали, что реакция с никелем пригодна не только для обнаружения следов элемента, попавщего в ткань извне, но и в тех случаях, когда элемент, под влиянием какого-либо патологического процесса (острое отравление окисью углерода), аккумулируется в сердечной мышце или освобождается и выходит из нее.

Наши исследования, выполненные с помощью энергия-дисперсионного микроанализа, единогласно подтверждают наличие никеля в продуктах реакции.

ВЛИЯНИЕ ВЕРАПАМИЛА И ФЕНОКСИБЕНЗАМИНА НА КОРОНАРНУЮ ВАЗОКОНСТРИКЦИЮ, ВЫЗВАННУЮ ИОНАМИ НИКЕЛЯ У НАРКОТИЗИРОВАННЫХ СОБАК

А. КОЛЛЕР, Г. РУБАНИ, Л. ЛИГЕТИ и А. ДЬ. Б. КОВАЧ

В настоящих экспериментах мы исследовали возможный механизм действия коронарной вазоконстрикции, возникающей в сердце собаки ***in situ*** под влиянием ионов никеля: 1. в присутствии селективного антагониста кальция верапамила и 2. в условиях блокирования альфа-адренергических рецепторов феноксибензамином (PBZ).

Верапамил полностью прекращал снижающее коронарный кровоток и базальную кондуктанцию действие низких доз (0,02—0,2 мг/кг) ионов никеля.

Действие более высоких доз (2,0—20,0 мг/кг) ионов никеля верапамил не только прекратил, но и извратил, то-есть, при наружном введении высоких доз $NiCl_2$, в присутствии селективного антагониста кальция, наблюдалось увеличение коронарного кровотока и повышени базальной кондуктанции.

Предварительная обработка феноксибензамином не оказывала достоверного влияния на эффект ионов никеля, проявляющийся в уменьшении коронарного кровотока.

Результаты экспериментов указывают на то, что вазоконстрикция, вызываемая в сердце собаки ***in situ*** добавлением во внешнюю среду микроколичеств хлористого никеля, наступает вследствие увеличивающегося поступления кальция в галдкомышечные клетки сосудов, которое передается не через посредство альфа-адренергических рецепторов.

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Physiologia — Pathophysiologia

ELECTRIC SIGN OF THE ACTIVITY OF THE SINUS NODE IN THE DOG HEART

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(Received September 15, 1981)

The possibility of local recording of the electrical activity of the sinus node was investigated on 22 dogs. Using a small unipolar electrode positioned at the proximity of the sinus node and applying a 2–4-fold electric amplification, an early, slow and low-amplitude wave could be recorded 20–40 ms before the P wave. This early wave gradually decreased when the electrode was moved away from the sinus node and finally fused into the local P wave. Temporary arrest of sinus node activity by injection of adenosine into the sinus node artery, electrical stimulation of the vagal nerve by infiltration with phenol of the sinus node region made the local P wave to disappear. Similarly, no early action before the P wave could be seen if the sinus node was driven electrically. The early wave recorded in the proximity of the sinus node represents the depolarization of the sinus node, and it is suitable to study the pacemaker activity of the sinus node together with sinoauricular conduction.

The sinus node is the driver, the pacemaker of cardiac rhythm. The cardiac cycle begins with the impulse originating in the sinus node. The activity of the sinus node has no electric sign in the ECG record. The first sign is the P wave that already means the spreading of the stimulus over the auricular muscle. In order to obtain more exact information on impulse generation and conduction in the heart, it would be of great importance to record the electrical activity of the sinus node or of the region adjacent to the node. The action potential generated in the sinus node cells is however too low for ECG recording. Still, in the last decade it has become possible to record the electric sign of the His bundle appropriately amplifying the local electrogram.

In the present experiments it was studied whether or not the electric sign of sinus node activity could be recognized in the proximity of the sinus node and whether there was any electric sign around the sinus node before the onset of the P wave.

Materials and Methods

The experiments were performed on 22 mongrel dogs of both sexes weighing 15 to 25 kg. Under sodium pentobarbital (30 mg/kg i.v.) anaesthesia and artificial respiration the chest was opened by transection of the sternum between the second and third ribs. After opening the pericardium the heart was opened widely to bring into sight the right auricle, the auricular part of the superior vena cava as well as the right atrium.

In pilot experiments four unipolar thick circular clamp electrodes were placed at the proximity of the sinus node, two at its border and two at the right auricle. Thereafter a unipolar electrode was sewed to the peak of the right auricle and another one to the left auricle. ECG tracings were recorded with a Hellige EK 26 three-channel direct writing recorder and with a Hellige 6-channel multiscriptor. A His preamplifier with high amplification was also used at a frequency range below 15 Hz.

Recording of the electrical activity of the sinus node

The experiments were performed on 15 dogs. Two electrodes of 8–12 mm^2 surface were sewed with atraumatic needle to the neighbourhood of the sinus node. Four further electrodes were applied, one near the sinus node on the right auricle, one more remotely, the third one on the left auricle, and the fourth one on the epicardiac surface of the right ventricle. The ECG was recorded by a Hellige multiscriptor at 2–4-fold amplification (1 mV = 20–40 mm) and 50–100 mm/s paper speed. The activity of the sinus node was arrested by the injection

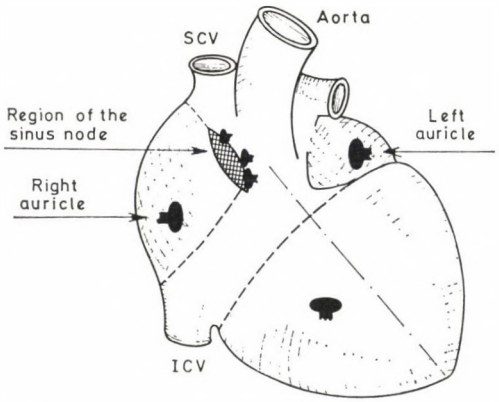

Fig. 1. Recording of the electrical activity of the sinus node in the dog. The three electrodes fixed to the neighbourhood of the sinus node serve to record the local early wave that follows sinus node activity. The electrodes sewed in the left atrium and right ventricle serve to record the epicardial electrogram of the left atrium and right ventricle.

of adenosine (100–200 μg) into sinus node artery in seven dogs. In five further animals sinus arrest was elicited by electrical stimulation of the vagal nerve (20 Hz, 1 ms, 6 V) and, in three further dogs, by injecting 10% phenol into the region of the sinus node. Artificial atrial pacemaker driving was produced in six experiments by means of quick electrical stimulation of the right or the left atrium. The experimental arrangement is shown in Fig. 1.

Results

In the pilot experiments a small wave that followed the sinus node activity could be recorded before the P wave in the neighbourhood of the sinus node, in the region of the right auricle and of the right atrium. The early wave was slow and small, and it preceded by 20–40 ms the beginning of the P wave, i.e. the local right atrial P wave. The wave was mostly positive except the electrogram recorded in the vicinity of the insertion of the superior

vena cava, where mainly a small negative wave was seen. Moving away from the proximity of the sinus node the early local wave became smaller and smaller until it gradually merged into the local P wave. No early wave could be recorded from the region of the left atrium and auricle. The early local wave preceding the P wave could permanently be recorded by means of the electrodes fixed to the proximity of the sinus node (Fig. 2).

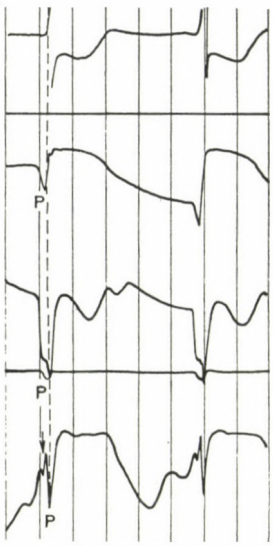

Fig. 2. Recording of the local activity of the sinus node from a region near the sinus node. Paper recording speed: 100 mm/s; 1 mV = 20 mm. Epicardial electrograms: top line = left ventricle; second line = right atrium; third line = right auricular region near the sinus node; bottom (fourth) line = immediate proximity of the sinus node. In the region near the sinus node an early wave, indicative of its activity could be seen 20–40 ms before the atrial P wave (↙). The right atrial P wave somewhat preceded the left atrial P wave

When impulse generation in the sinus node was suppressed and the right atrium was driven electrically, no local early wave appeared before the P wave that followed the artificial spike of electric stimulation (Fig. 3).

Impulse initiation in the sinus node could transiently be abolished by vagal stimulation or by injection of adenosine into the artery leading to the sinus node. When the sinus node activity ceased the accompanying local early wave disappeared (Fig. 4).

Similarly, disappearance of the local early wave and deformation of the P wave were observed after phenol infiltration, stimulus generation then seemed to originate from the right atrium instead of the sinus node.

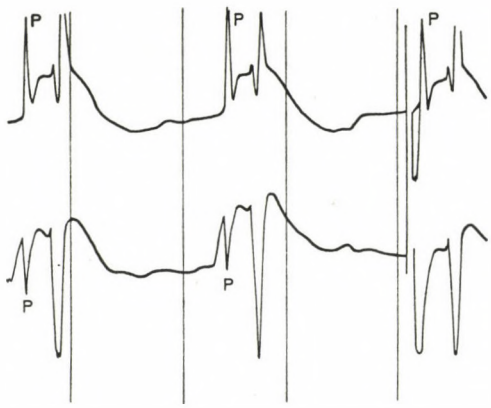

Fig. 3. Effect of electric stimulation of the sinus node on the local early wave. Paper recording speed: 100 mm/s; 1 mV = 25 mm. Epicardial electrograms: upper line = right auricle; bottom line = proximity of the sinus node. During the activity of the sinus node, an early wave (↙) could be recorded from its proximity. During electrical stimulation of the right atrium (third beat) no early wave can be seen prior to the P wave; the spike (S) is immediately followed by the P wave and then by the R wave

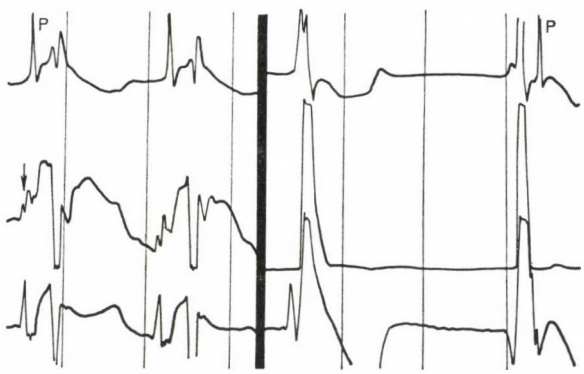

Fig. 4. Alteration of the local early wave of the sinus node after local application of adenosine. Paper recording speed: 100 mm/s; 1 mV = 20 mm. Epicardial electrograms: upper line = left atrium; middle line = immediate proximity of the sinus node; bottom line = right auricle. The record on the left was taken during the activity of the sinus node: a well visible early wave appears in the middle line (record from a region near the sinus node). Record on the right was taken after injecting adenosine: as an effect, marked bradycardia and junctional rhythm appear. No early local wave can be seen before the P wave

Discussion

Recording of sinus node depolarization would greatly promote the study of cardiac arrhythmias. Recording of the activity of the sinus node or of its surroundings would allow to distinguish a sinoatrial block from a sinus arrest due to the lack of impulse initiation in the sinus node. It would also

allow to differentiate between a tachycardia originating in the sinus node and in the atrium. A sinus node re-entry tachycardia would also be recognized. Recording of the early wave preceding the P wave and indicative of the activity of the sinus node allows the exact measurement of the sinoauricular conduction time. Up to now the conduction time from sinus node to atrium has been measured in an indirect way, by applying an early atrial stimulus (NARULA et al. [11]; STRAUSS et al. [13]). Neither of these methods is reliable, and the application of early atrial stimulus is not even theoretically appropriate to measure the real sinoatrial conduction time (LU et al. [10]; BONKE et al. [3]; BOUMAN et al. [4]; ANDERSON et al. [1]).

Several different attempts have been made to record the activity of the sinus node. The first procedure consisted in recording the action potential of the sinus node in perfused sinus node — right atrium preparation (BONKE et al. [3]; BOUMAN et al. [4]; DORTICOS et al. [7]) The method is technically difficult and has the drawback that it does not allow recording on the beating heart.

For the analysis of arrhythmias one or several electrodes are introduced into the right atrium. The electrodes are directed towards and fixed to the corner of the superior vena cava and the right atrium; under such conditions, the atrial ECG can be led from the immediate proximity of the sinus node. By appropriate amplification, several authors succeeded in recording a wave accompanying early sinus node activity and appearing before the P wave (CASTILLO et al. [5]; HOMBACH et al. [9]; HARIMAN et al. [8]; BETHGE et al. [2]; PASAPIA et al. [12]). It is rather difficult to place and fix the electrode near the sinus node; therefore, the measurements are not always reliable and not always reproducible.

The third method consists in sewing the electrodes in the neighbourhood of the sinus node and recording the local, early, waves. CRAMER et al. [6] mainly used bipolar electrodes in their dog experiments, overbridging thereby the territory of the sinus node. They could record a local activity before the P wave similarly to the early wave recorded in our present experiments.

The present results together with the mentioned data in the literature showed that with an appropriate technique a local early wave corresponding to the activity of the sinus node can be recorded in its proximity. This wave is a reliable indicator of the electrical activity of the sinus node. Arrest of the sinus node activity by adenosine, vagal stimulation or phenol infiltration leads to disappearance of the early wave. Similarly, no early wave can be recorded when the atrium is driven electrically.

Moving away from the sinus node the early wave becomes less and less recordable; finally, it disappears, i.e. merges into the P wave (Fig. 5). The sinoauricular conduction time is short in the dog, about 20–40 ms under normal conditions. Systematic recording of the close local activity of the sinus

node and improvement of the technique of such recording will allow an exact study of both the pacemaker activity and the auricular stimulus conduction of the sinus node.

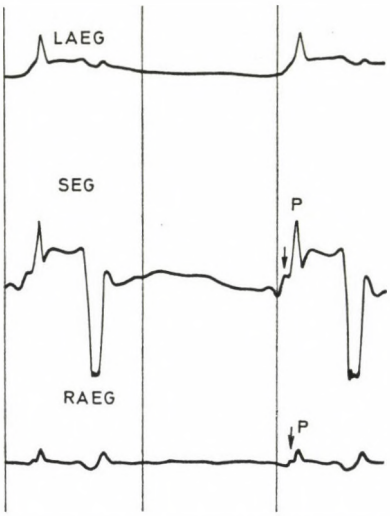

Fig. 5. The change of the position of the local early wave of the sinus node. Paper recording speed: 100 mm/s; 1 mV = 20 mm. Epicardial electrograms: top line = left atrium; middle line = immediate proximity of the sinus node; bottom line = right atrium, 6 cm from the sinus node region. Early wave (↙) can be detected before the atrial P wave in the sinus node region. The early wave will be melted into the local—right atrial—P wave by moving away from the sinus node region]

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THE ROLE OF THE MYOCARDIAL WATER CONTENT IN HEART FUNCTION

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(Received October 26, 1981)

In previous experiments a considerable interstitial oedema developed after myocardial infarction followed by the infusion of 6 mmol/kg/min norepinephrine lasting for 10 min in the 2nd and 48th hours as well as after a 60 min period of ventricular fibrillation during cardiopulmonary bypass. On the other hand myocardial dehydration was induced by hyperosmolality of 320–470 mOsm/l caused by mannitol or glucose after pancreatectomy. A close bilinear correlation was found between the myocardial water content and the increase of ventricular diastolic stiffness. It was further established that the increase of ventricular stiffness is followed by a decrease of the cardiac output index measured during left ventricular afterload. The cardiac output index was lower after myocardial infarction and pancreatectomy than during cardiopulmonary bypass or mannitol-treatment. Of clinical importance is the fact that ventricular performance decreases when myocardial water content exceeds or does not reach the value of 760–790 g water per 1000 g myocardium.

Elevated diastolic stiffness of the ventricular wall plays an important role in a number of heart diseases such as asymmetric septum hypertrophy [8, 9], diabetic cardiomyopathy [11, 18], angina pectoris [1] and myocardial infarction [2, 3, 5, 10]. Recently we have shown that left ventricular diastolic stiffness is elevated in myocardial oedema [12, 13, 14] and myocardial dehydration [15, 16, 17]. The aim of the present study was therefore to demonstrate a universally valid correlation between tissue water content and diastolic stiffness in the myocardium as well as between the latter and ventricular performance.

Methods

In the study 45 mongrel dogs of both sexes weighing 10–25 kg were used. Myocardial dehydration was induced by 11 mmol/kg glucose given in 2 or 3 consecutive intravenous injections in 6 pancreatectomized animals. This dose of glucose induced a hyperosmolality ranging between 320–470 mOsm/l. Since insulin influences the dehydrating effect of glucose, [16] pancreatectomy was performed in these (n = 10) animals. Haemodynamic investigations were carried out half an hour after pancreatectomy. In another group of animals hyperosmolality of 320–470 mOsm/l was induced by 2.745–5.49 mmol/kg of mannitol [16]. Myocardial oedema was induced partly by i.v. infusion of 6 nmol/min/kg norepinephrine for 10 min, performed 2 or 48 hours after ligation of the left anterior descending coronary artery in 7 and 6 animals, respectively, and partly by empty beating or fibrillating hearts during cardiopulmonary bypass for 60 min in 17 animals. The data from 8 intact animals served as controls.

Myocardical water content was determined by drying the tissue to constant weight and expressed as a percentage of the total wet weight. The diastolic stiffness of the left ventricular wall was characterized by the value for the left ventricular passive elastic modulus,

which was calculated by the modified method of DIAMOND and FORRESTER [2]. This modification by gradual blocking of the flow in the descending aorta by a step-by-step inflated embolectomy catheter introduced through the femoral artery allowed to measure the correlation of $\Delta P/\Delta V$ and the mean intraventricular diastolic pressure in a wide range [6]. Since the pressure–volume relationship is not exponential at low pressure, data obtained at a pressure of less than 333 Pa were neglected. Pressures were measured with Statham gauges (p23Db) through rigid catheters introduced into the ascending aorta and the left ventricular apex. Cardiac output was measured by the thermodilution method [4]. The calculations were carried out using linear and bilinear regression analysis. The latter was calculated by the method of KUBINYI and KEHRHAHN [7].

Results

Comparing the data of animals with myocardial oedema to those of the controls, a close linear correlation ($y = 0.0358x - 2.6939$; $r = 0.6247$; $n = 41$) was found between the passive elastic modulus and the myocardial water content in the left ventricular wall. Thus the increase of the myocardial water content is followed by a proportional elevation of the value of the passive elastic modulus. On the other hand, by pooling the data obtained in myocardial dehydration and in the control state, an inverse linear correlation ($y = 0.0448x + 3.5502$; $r = 0.9405$; $n = 24$) could be demonstrated between the above parameters. The decrease in myocardial water content increased the values of the left ventricular passive elastic modulus. Considering the diverse alterations of myocardial water content, a close bilinear correlation was observed between the tissue water content and the passive elastic modulus (Fig. 1). This phenomenon is referring to the dependence of myocardial diastolic elasticity on the myocardial water content. As shown in Fig. 1, the smallest diastolic stiffness of the myocardial wall coincides with a normal tissue water content and myocardial diastolic stiffness, characterized by the values of passive elastic modulus, increases immediately when the tissue water content deviates from the normal level.

The functional state of the myocardium was characterized in all experimental groups by the cardiac output index measured during left ventricular afterload, i.e. when the flow was blocked by means of an inflated embolectomy catheter (Table I). Left ventricular performance was significantly decreased in all experimental groups except in the animals with empty beating heart during cardiopulmonary bypass. This could be explained by the fact that their myocardial contractility was damaged to a lesser extent and the interstitial oedema was slight in these cases. The correlation between the passive elastic modulus, characteristic of left ventricular diastolic stiffness, and the cardiac output index during left ventricular afterload demonstrated that the cardiac output index always diminished when the left ventricular diastolic stiffness increased (Fig. 2). As shown in Fig. 2, two curves for correlation—both including the control dogs—had to be taken separately among the experimental animals. Several groups (two with myocardial infarction and the

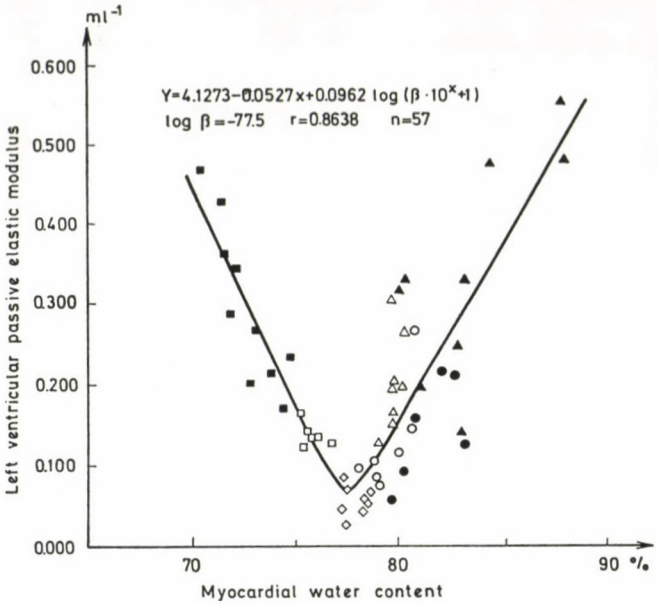

Fig. 1. Bilinear correlation between tissue water content and passive elastic modulus in the left ventricular myocardium. ◇ control animals; □ animals with glucose-induced hyperosmolality after pancreatectomy; ■ animals with mannitol-induced hyperosmolality; ○ animals treated with norepinephrine 2 hours after coronary artery ligation; ● animals treated with norepinephrine 48 hours after coronary artery ligation; △ animals with empty beating hearts during cardiopulmonary bypass; ▲ animals with empty fibrillating hearts during cardiopulmonary bypass

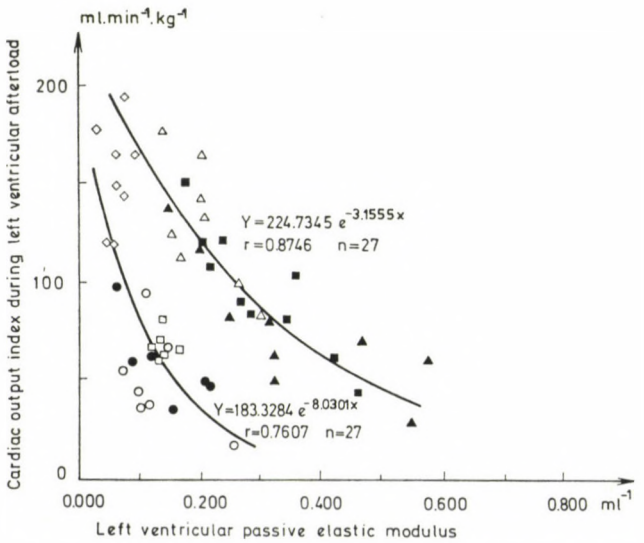

Fig. 2. Influence of ventricular diastolic stiffness on the cardiac output index during left ventricular afterload. Legends see in Fig. 1

Table I

Cardiac output index from all experimental groups of the study

Groups	Cardiac output index during left ventricular afterload	n
◇	153 ± 9^b	8
□	46 ± 7^b	6
■	57 ± 8^b	10
○	70 ± 3^b	7
●	94 ± 10^a	6
△	126 ± 14^b	8
▲	73 ± 4^b	9

n = number of animals

The significance relative to control values is indicated by a: $p < 0.01$; b: $p < 0.001$ Symbols see in Fig. 1

pancreatectomized one with glucose-induced hyperosmolality) showed a lower cardiac output index than did the remaining groups. According to the figure, no difference in cardiac output index was found between the two coronary infarction groups, independently of the fact that norepinephrine was administered 2 or 48 hours after the ligation.

Discussion

It is a common feature of biological phenomena that the optimum function of an organ is ensured solely when important characteristics do not deviate from their normal levels. This kind of correlation could also be demonstrated in our observation between tissue water content and myocardial diastolic stiffness.

Our earlier investigations [12, 13, 14, 15, 16, 17] have confirmed the observation of SKELTON [19] and have shown that myocardial water content normally ranges between extremely close limits and 100 g of wet myocardium contains under normal circumstances 77.8 ± 1.0 g water. This indicates that the normal tissue water content is maintained in the myocardium by a very sensitive regulatory mechanism. According to our findings the value of left ventricular passive elastic modulus increases and accordingly the compliance of the left ventricular wall decreases when the myocardial water content exceeds or does not reach the values of 760–790 g in 1000 g myocardium, independently of how an elevation of the left ventricular diastolic stiffness could be reached. We determined only the water content of the myocardium, and in this way only the fact of oedema could be observed, without any possibility to judge whether the different interventions on the extra- or intra-

cellular space exerted their effects predominantly. This phenomenon is of clinical importance since ventricular performance, i.e. the cardiac output index has been found to decrease when the myocardial water content was higher or lower than its normal level. In such conditions namely the values of the passive elastic modulus are elevated [11, 13, 14, 15, 16, 17].

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COMPARATIVE STUDY OF TRANSITION TEMPERATURE IN ADULT AND NEWBORN HUMAN RED BLOOD CELLS

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Hypotonic haemolysis of newborn and adult human red blood cells (RBC) is more intensive at 4 than at 37 °C. Newborn RBC are more resistant to the decrease of temperature than adult RBC. Transition temperature of the RBC membrane can well be determined by illustrating osmotic haemolysis as a function of temperature. This parameter characterizes well the microviscosity of the membrane. The inclination point of Arrhenius curves representing transition temperature occurred always at 25 °C both in the case of adult and newborn RBC. The similarity of the transition temperature indicates the similarity of membrane microviscosity of newborn and adult human RBC.

Surface components of the adult human red blood cell (RBC) membrane are practically immobile in the plane of the membrane [9]. Antibodies and lectins bind to the surface receptors of the normal RBC, but in contrast to other cells, they do not induce redistribution or endocytosis of these receptors [3].

The properties of human RBC are different in the newborn. Endocytosis can be provoked in newborn RBC by antibodies specific for blood group "A" [2]. This finding agrees well with that of SCHEKMAN and SINGER who found that lateral mobility of certain receptors and antigens is higher in the newborn than in the adult cell [11].

The question arises what can be the cause of these differences found in the lateral mobility of the receptors. One possible explanation is the lower viscosity of the lipid layer in the newborn cell membrane. The temperature dependence of the osmotic resistance of RBC was studied in one of our previous works [7]. The illustration of osmotic haemolysis as the function of temperature made the determination of transition temperature possible. Microviscosity of the membrane can be well characterized by this parameter. The lower viscosity of the young calf RBC membrane compared to adult cattle RBC could be verified by this method.

The present experiments were designed to study the transition temperature of human RBC in newborns and adults by analysing the temperature dependence of the osmotic resistance of RBC.

Materials and Methods

Red blood cells (RBCs) removed from 10 healthy newborn babies and 15 adult men and women (20 to 60 years old) were washed [5] and 0.1 ml portions of their 30% solution were added to a series of hypotonic solution, 4 ml each. Hypotonic solutions of 210, 190, 170, 150, 130 and 110 imOsm osmotic strength were prepared from a NaCl solution containing 10 mM Veronal Na—HCl buffer (pH 7.4). Incubation was carried out for 15 min at 4, 9, 16, 25, 30 and 37 °C. After incubation the mixture was centrifuged at 1000 *g* for 3 min at 0–4 °C, and the haemoglobin content of the supernatant was determined by a spectrophotometer at 540 nm. The extent of haemolysis was expressed as per cent of total haemolysis obtained after treatment of the RBC suspension by Triton X-100 (0.3 mg/ml).

Results

Hypotonic haemolysis of both the newborn and adult RBCs was more pronounced at 4 than at 37 °C (Figs 1 and 2). The shape of the haemolysis curve was very similar in newborns and adults, which was obvious also from

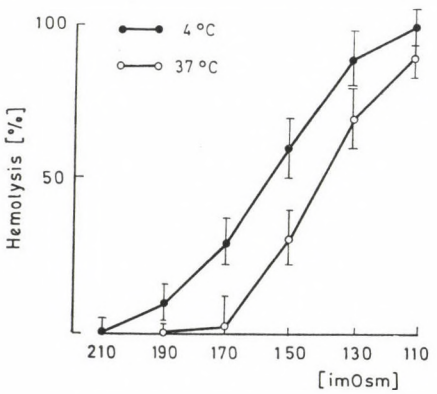

Fig. 1. Osmotic haemolysis of newborn human red blood cells at 4 and 37 °C. Haemolysis expressed as per cent of total haemolysis and illustrated as the function of osmotic strength of the NaCl solution

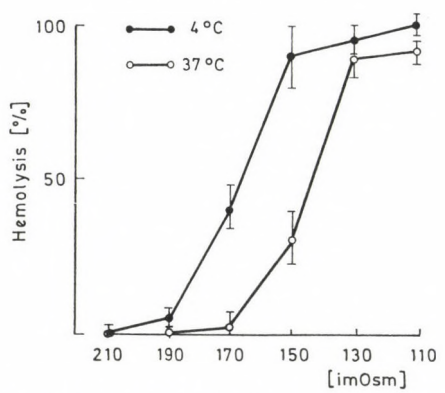

Fig. 2. Osmotic haemolysis of adult red blood cells at 4 and 37 °C. Haemolysis expressed as per cent of total haemolysis and illustrated as the function of osmotic strength of the NaCl solution

the fact that there was no great difference between the haemolysis value at 4 and 37 °C at both ends of the curve (i.e. at 210 and 110 imOsm osmotic strength). In other words the effect of temperature was negligible at minimum and maximum haemolytic rates.

Analysing the intermediate part of the curve (150 imOsm) it is essential to note that the difference between the haemolytic rates at 4 and 37 °C was significantly more pronounced in adult RBC (Fig. 3). This difference could be explained by the fact that newborn RBCs were more resistant to the decrease of temperature during hypotonic haemolysis than adult RBC.

Illustrating the haemolytic rate as the function of temperature in 150 imOsm NaCl solution, Arrhenius lines could be constructed (Fig. 4). Inflection points of these lines occurred at 25 °C both in the case of newborn and adult RBC.

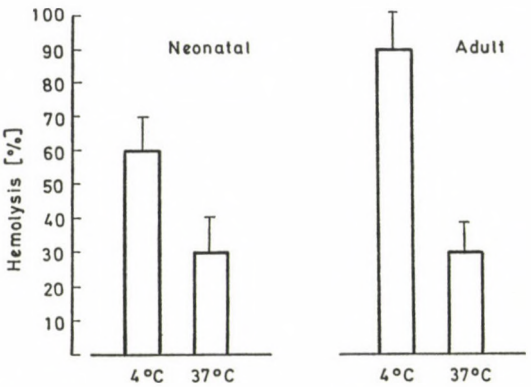

Fig. 3. Haemolysis of newborn and adult human red blood cells at 4 and 37 °C in 150 imOsm NaCl solution. Haemolysis expressed as per cent of total haemolysis

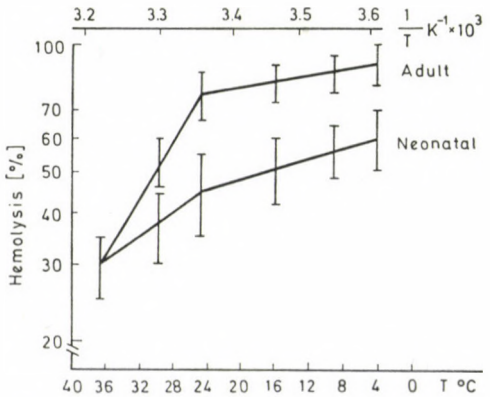

Fig. 4. Effect of temperature on osmotic haemolysis of newborn and adult human red blood cells in 150 imOsm NaCl solution. Haemolysis expressed as per cent of total haemolysis and as a function of incubation temperature

Discussion

The greater mobility of membrane receptors and antigens suggest lower microviscosity of the newborn RBC membrane. Previous studies revealed a lower transition temperature of calf RBCs than of adult cattle RBCs [7]. Lower transition temperature indicates reduced microviscosity.

KEHRY et al. [8] compared the microviscosity of the newborn and adult RBC membranes by fluorescence polarization technique. Their experiments showed negative results, i.e. there was no difference in the microviscosity of the two membranes. Our results are not in contradiction with those of KEHRY et al. Although the higher osmotic resistance of newborn RBCs at low temperature indicates increased membrane fluidity, the transition temperature—characterizing microviscosity—was similar in the newborn and adult RBCs.

ALONI et al. [1] found with the same technique that transition temperature of adult RBCs was 25 °C; they did not study newborn RBCs.

It is very difficult to explain that the microviscosity, measured either by fluorescence or by osmotic resistance technique, was not different in the cases of newborn and adult RBCs.

KEHRY et al. criticized their own method. According to their opinion the fluorescence probe by perylene gives information only about the state of the interior hydrocarbon layer and it is not suitable for the measurement of microviscosity of the whole membrane and especially of its surface layers.

In contrast to the old concept explaining microviscosity only on the basis of the lipid composition of membranes, the new concept involves the following factors as well: lipid composition, lipid–protein interactions, phosphorylation state and conformation of ATP-dependent membrane proteins and the presence of divalent cations [12].

Similar to ALONI we also believe that the analysis of the temperature dependence of osmotic resistance provides valuable information about the microviscosity of the protein–lipid boundary region rather than about that of the inner lipid layer. In this case cellular ATP content and Mg/Ca ratio are of particular importance [12]. The 10 times higher ATP content [6] and the higher Mg^{2+} concentration [5] of the newborn calf RBC may explain the lower microviscosity of the protein–lipid boundary region [7]. Such a great difference in ATP or Mg content of newborn and adult human RBCs has not been found so far [10, 4].

This fact may explain that, without further development of the technique, no difference will be found between the transition temperature, i.e. microviscosity of the lipid–protein boundary region, of adult and newborn RBCs.

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COMPARATIVE STUDY ON THE ACUTE EFFECTS OF BENZENE, TOLUENE AND m-XYLENE IN THE RAT

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Solvents were tested for their influence on motivational state, as well as for their sedative effects, after intraperitoneal administration. The following tests were used: open field (exploratory activity, locomotion), activity wheel (running activity), tube test and tilted plane (muscle weakness, incoordination).

The agents proved to be similar in causing muscle weakness and ataxy already at low dose levels. Benzene was the most potent in this respect. The three solvents, however, contrasted strongly in influencing motor activity. While benzene and especially toluene exerted CNS stimulation, no such effect could be detected following m-xylene treatment. On the basis of human experiences with these substances it may be suggested that the screening procedure applied in rats might be useful for predicting acute adverse effects in man.

At the time being industrial solvents are generally known as central nervous system depressants. Depending upon the level of exposure, however, there are certain differences in the CNS profile of the substances. This fact might be of great importance not only concerning the hygienic aspects but also the increasing abuse of these agents ("glue sniffing"). Inhalation of toluene preceding narcosis is known to cause euphoria, exhilaration, excitement and drunkenness [9, 10, 2], while no such experiences are available with xylene exposure.

A revision of our knowledge about the CNS effects of solvents is required and there is a need to find suitable methods to differentiate among solvents regarding their acute effects in animals. Taking into consideration that the acute effects of organic solvents are transitory owing to their rapid elimination, a test battery was developed in our laboratory for rats which allows the analysis of motivational states as well as the assessment of certain sedative effects, such as incoordination and muscle weakness, within a very short time. The screening battery consists of several simple tasks possible to complete without training procedure. The validity of the method was proved by three commonly used aromatic solvents, applied intraperitoneally.

Methods

Animals. CFY male white rats obtained from the LÁTI (Gödöllő, Hungary) breeding colony were housed in groups of ten in wire mesh cages of $36 \times 30 \times 50$ cm. A standard rat diet in pellets and tap water were available ad libitum. Experiments were performed in ani-

mals reaching an age of 8 weeks and weighing about 200 g. Rats were transferred to the laboratory 30 min before the experiments, always between 8.30 and 11.30 a.m.

Materials, dosage. Benzene (Biogal, Hungary), toluene (Biogal), and m-xylene (Reachim, USSR) with declared high analytical purity was used. The agents were diluted with sunflower oil and injected intraperitoneally 30 min prior to the experimental session. Control animals received the vehicle alone. At least five dose levels for each compound were tested. Statistical evaluation was performed with Student's t test.

The tests applied were as follows:

Modified open field. The apparatus consisted of a circular flat area (45 cm in diameter) enclosed with a cardboard wall (25 cm high), where the animal could move freely on the periphery but was prevented from crossing the middle part of the field by a centrally placed cylinder (15 cm in diameter and 25 cm high). The floor between the inner and outer wall was divided into 8 segments, and the whole area was illuminated by a 100 W lamp 100 cm above the floor. The apparatus was located in a quiet room and the rats were watched by means of mirrors. The two parameters registered in the five minute test period were: ambulation indicating locomotor activity (the number of segments crossed), and rearing indicating vertical orientation.

Activity wheel. The wheel was made of perforated Plexiglass (28 cm in diameter and 5 cm wide). Revolutions were counted by a mechanical system for 30 min.

Tilted plane (1). Rats were placed onto the rough surface of a wooden plane (45 × 70 cm) inclined at an angle of 30 degrees. The plane was tilted continuously until the rat slid down. The angle of the tilted plane was registered. The mean value of three trials was given.

Tube test. A rat was made walk inside a tube (a dark, round box, 13 cm long and 7.4 cm in diameter) in horizontal position. Then the tube was quickly turned into vertical direction with the rat's head upside down. An untreated rat corrected its unusual position 3–5 s. Maximum waiting time: 20 s.

Results

Dose–response curves for the three solvents obtained by the different tests are presented in Figs 1 through 5.

Open-field behaviour (Figs 1 and 2). There was a striking difference among the action of drugs tested. Depending on the dose applied toluene exerted two opposite effects. In a certain dose range a dose-dependent increase

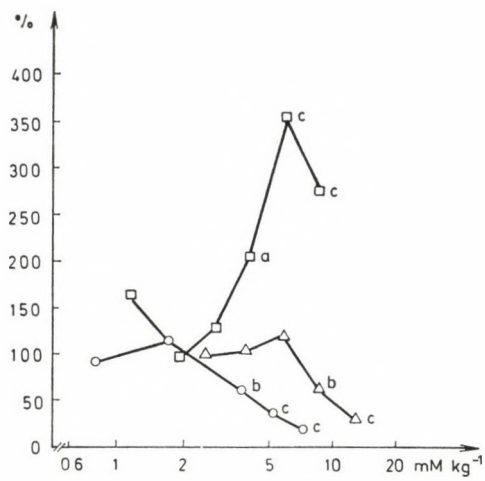

Fig. 1. Open field, ambulatory activity. Control, 100%. The data represent the mean values of ten rats. ○ benzene, □ toluene, △ m-xylene. Level of significance: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$

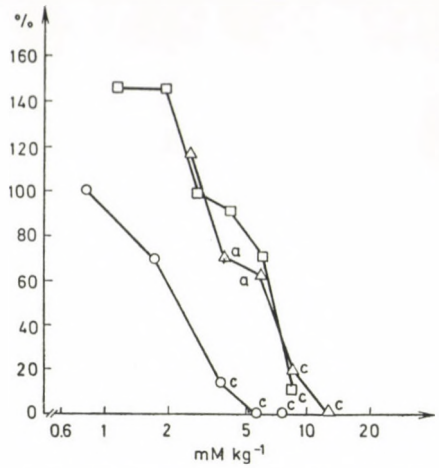

Fig. 2. Open field, rearing activity. Control, 100%. Symbols as in Fig. 1

in locomotor activity could be recorded and at lower dose levels rearing activity was also enhanced. Higher doses caused depression in both tests. On the other hand, depression was the only effect which could be detected after the administration of benzene and m-xylene.

Wheel-running activity (Fig. 3). Under the influence of toluene the increased open-field ambulation was accompanied by increased wheel running.

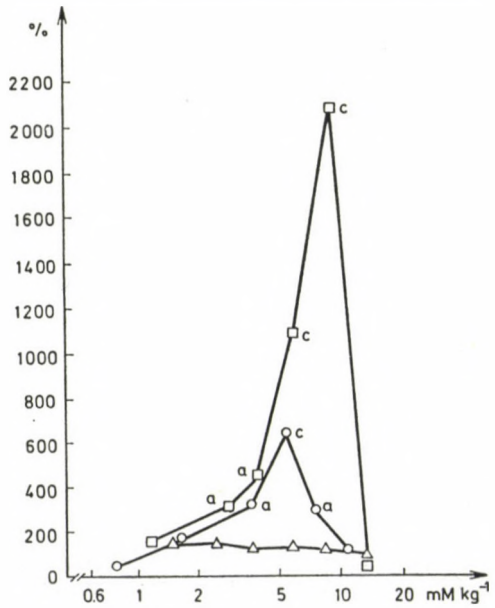

Fig. 3. Performance on the activity wheel. Control, 100%. Symbols as in Fig. 1

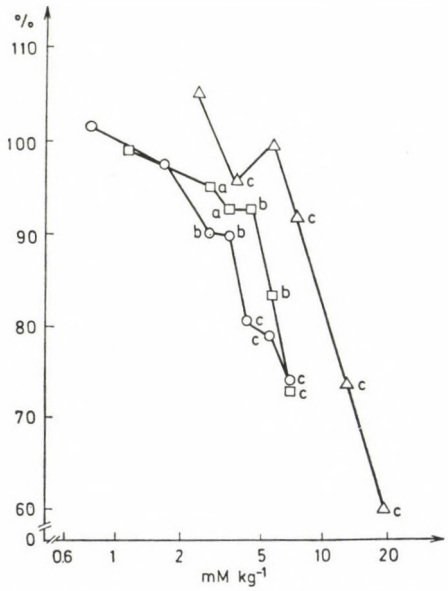

Fig. 4. Performance on the tilted plane. Control, 100%. Symbols as in Fig. 1

Benzene also increased wheel running up to a certain degree, while m-xylene had no effect at all.

Tilted plane and tube test (Figs 4 and 5). All the three solvents caused muscular weakness, ataxy and incoordination interfering with the performance of the animals in both tests. The angle of the tilted plane at which rats slid down as well as the time required for rats to correct their inverse position in the tube changed in a dose-dependent manner.

While the relative potency of solvents can be assessed in Figs 1 through 5, in Table I the lowest dose ranges evoking significant changes in the test

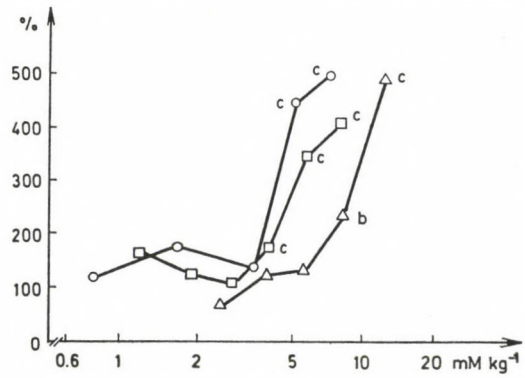

Fig. 5. Performance in the tube test. Control, 100%. Symbols as in Fig. 1

Table I

Minimum effective dose ranges determined by the test battery 30 min after the administration of solvents

Solvent \ Test	Minimum effective dose ranges (mM/kg)					Acute LD_{50} values (95% confidence intervals)
	Open field, ambulation	Open field, rearing	Tube test	Tilted plane	Activity wheel	
Benzene	1.7—3.6** ↓	1.7—3.6** ↓	3.6—5.2** ↓	1.7—2.8** ↓	1.7—3.6* ↑	23.15 (19.35–27.74)
Toluene	2.7—4.0* ↑	5.8—8.3** ↓	2.7—4.0** ↓	1.1—2.8* ↓	1.1—2.7* ↑	23.39 (20.49–25.57)
m-Xylene	5.6—8.4** ↓	2.5—3.7** ↓	5.6—8.4** ↓	2.5—3.7** ↓	no effect	21.06 (18.87–23.49)

The upper limit values of the minimum effective dose ranges represent significant differences from the controls; *$p < 0.05$; **$p < 0.01$; ↑ increased activity; ↓ decreased activity

performances can be compared with the LD_{50} values also determined in our laboratory (unpublished data).

Discussion

With the aid of the testing procedure we succeeded in finding some features of the three substances investigated being *in common*, as well as features of completely *different* character.

Benzene, toluene, as well as m-xylene caused muscular weakness and equilibrium disturbances. These common symptoms associated with the well-known sedative effects of industrial solvents were detected by sensory-motor methods, such as tilted plane, tube test and at the same doses by the open-field rearing activity. In this respect benzene was the most potent, probably because the intensive tremor appearing as a special symptom following its injections might have influenced the test performance.

Considering motivational states, however, toluene differed from the other solvents qualitatively and quantitatively as well. Toluene seems to be a central nervous system stimulant. This stimulatory action can be observed already at small doses and in a wide range in the open field ambulation and in the activity wheel. Benzene also enhanced wheel running activity up to a certain degree, the intensive tremor, however, might have suppressed the manifestation of the CNS excitation in the wheel. No sign of excitation following m-xylene treatment could be detected, the decreased test performances reflected the predominance of a CNS depressant effect.

Concerning the effects of toluene, however, we have to stress that the excitement measured in the wheel and in the open field at low doses is a phenomenon different from the marked activities at high doses. While at lower doses activity reflects a stimulated exploration (increased ambulation *and* rearing), the increased locomotor activity at higher doses and the absence of vertical orientation (rearing) probably represents stereotypy. This assumption can be confirmed by the wheel-running behaviour at high doses, where animals with a high degree of ataxy and muscular weakness were able to run the wheel nearly non-stop with a maximum speed. This high activity does not represent muscular strength (wheels are easy to drive), on the contrary, while in the activity wheel a drive of maximum speed can be recorded, the performance on the tilted plane or in the tube might be rather poor. This type of drug action is not new; e.g. Cook et al. [4] found that pentobarbital stimulated motor activity even following doses that produced marked ataxia.

Although extrapolation of animal experimentation to predictability in man must be done carefully, we assume that the analysis of the unlearned behaviour under the effect of aromatics might be useful for the classification of solvents in respect of their acute adverse effects in man. The presence or

absence of the excitatory component in the actions seems to be decisive in the assessment.

Thus, m-xylene exerted no stimulatory effect on the CNS in the rats. Similarly, data for an excitatory action in humans are also lacking [6]. Benzene, and especially toluene produced CNS stimulation in experimental animals. This was in accord with the expectations, since both solvents are known to cause euphoria in man [3, 6, 7]. Moreover, the pronounced excitatory effect of toluene in the rat resembles that of psychomimetics. According to HORVÁTH and FRANTIK [8], substances increasing spontaneous motor activity in lower and medium concentrations and depressing it only in very high ones evoke euphoria in man. DAMSTRA [5] classified toluene as a hallucinogen and Japanese authors [11] studying the changes in the EEG components after toluene exposure suggest that the persistence of the rhythmical hippocampal waves may have some common bearings with the psychomimetic effect of toluene in cats. Toluene was studied by WEISS et al. [12] for its capacity to maintain self-administration of monkeys in the same way as drugs of abuse.

The test battery capable of measuring different levels of excitation and/or depression proved to be appropriate for screening the acute effects of solvents following their intraperitoneal administration. The sensitivity of the method can be judged from the data presented in Table I. There are about five to tenfold differences in the minimal effective doses and LD_{50} values. It is reasonable to suppose that similar actions as well as similar differences in doses (concentrations) are to be expected in inhalation studies. This is, however, going to be the topic of a subsequent study.

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UTERO-INHIBIN: A NEW SUBSTANCE INHIBITING UTERINE CONTRACTION, ISOLATED FROM AMNIOTIC FLUID

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Amniotic fluid was collected from women in the 2nd trimester of their gravidity by amniocentesis and its effect on the isolated pregnant rat uterine preparation investigated. It was demonstrated that some of the amniotic fluid components partially purified by using the combination of various separation techniques stimulated, while others inhibited the contractions of the isolated rat uterine preparation. The inhibitory component, called utero-inhibin also inhibited the spontaneous contractions as well as the basal tone of the isolated rat intestinal preparation and those of the isolated human pregnant myometrium preparation. It is speculated that the inhibitory factor might play a physiological role in maintaninig the resting state of human uterus during pregnancy.

Knowledge concerning the factors controlling human myometrial function during pregnancy and parturition is incomplete in spite of numerous experimental observations. Experiments in rats and rabbits indicated the existence of a factor different from progesterone, inhibiting uterine motility during pregnancy [3, 6, 7]. The physiological and pathological significance as well as the physico-chemical and chemical nature of this (these) factor(s) have not been clarified.

The present study was aimed at clarifying the presence of a factor in human pregnancy capable of inhibiting uterine motility.

Materials and Methods

Collection and storage of specimens

Amniotic fluid was obtained by amniocentesis from healthy 16–22 weeks pregnant patients (n = 7). The specimens were immediately centrifuged at 4000 rpm for 20 min at room temperature. The supernatants were stored at −20 °C and a pool was prepared from them immediately before analysis.

Physico-chemical analysis

a) Sephadex G-25 fine (SG-25) gel chromatography. Column: 1.6×40 cm; eluent: Krebs solution; flow rate: 0.5 ml min^{-1}; room temperature; sample volume: 5.0 ml; transmittance: detected at 254 nm; calibration of the column: blue dextran (V_0) and acetone (V_{eff}).

b) CM-Sephadex C-25 (CM-SC-25) ion exchange chromatography. Column: 1.6×36 cm; eluents: 144 ml 0.05 and 144 ml 0.1 M HCl; room temperature. The fraction eluted by 0.1 M HCl was evaporated to dryness at 40 °C under reduced pressure and dissolved in distilled water.

c) This fraction was further separated by one-dimension paper chromatography. Whatman 3M paper; butanol : pyridine : glacial acetic acid : water = 30 : 40 : 6 : 15 v/v.

Biological analysis

Biological activities of the native and fractionated amniotic fluids were tested on strips of rat uterine horn removed in the 20th day of pregnancy and of human myometrium excised in the 40th week of pregnancy by Caesarean section, and on *in vitro* preparations of rat duodenum, ileum, colon ascendens as well as electrically driven (impulses of 2.2 msec duration, 70 V amplitude, at 12 s intervals) mouse vas deferens.

The experimental conditions were as follows.

1. Superfusion system (bath volume: 1.2 ml; flow rate: 0.5 ml min^{-1}; medium: Krebs solution; equilibration period: 90 min; pretension: 2.0 g; organ preparation: rat uterus).

2. Perfusion system (bath volume: 4.6 ml; medium: Krebs solution; temperature: 37 °C but the mouse vas deferens where 30 °C; pretension: at rat and human uterine strips 2.0 g, at rat intestine preparations 0.5 g and at mouse vas deferens 0.3 g; equilibration period: at uterine strips 90 min and at all other preparations 30 min). Continuous bubbling with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide and isometric recording (Bioforcemeter BFS 8) were used in both systems.

Chemical analysis

Peptide concentration of the native and fractionated amniotic fluids was determined by the modified LOWRY method [5].

Results

0.1 to 0.5 ml pooled human amniotic fluid (peptide concentration: 5.43×10^{-3} g ml^{-1}) obtained in the 16–22nd week of pregnancy increased the frequency and basal tone but decreased the amplitude of spontaneous contractions of the *in vitro* superfused pregnant rat uterine strip.

Native amniotic fluid could be separated by SG-25 chromatography into 3 fractions with effect on pregnant rat uterine strip. These fractions were characterized with the K_{av} values ($K_{av} = (V_e - V_0)(V_t - V_0)^{-1}$, where V_e = elution volume of individual fractions, V_0 = exclusion volume of the gel column, V_t = total volume of the gel column on the basis of geometrical parameters) determined at the maximum of biological activity. The first one of them ($K_{av} = 0.63$) inhibited the spontaneous contractions, the second one increased the frequency of contraction and basal tone ($K_{av} = 0.82$), the third one ($K_{av} = 0.93$) reduced the amplitude of contractions (Fig. 1). These fractions contained Folin positive materials and absorbed light at 254 nm wavelength. The $K_{av} = 0.63$ fraction was eluted within the effective separation volume (V_{eff}) of the gel column.

The subfraction of the $K_{av} = 0.63$ fraction eluted by 0.1 M HCl on CM-SC-25 cation exchange resin, inhibited spontaneous contractions and decreased the basal tone of the *in vitro* pregnant rat as well as human myometrium in a dose-dependent manner, in the range of 1.5 to 7.2×10^{-7} g ml^{-1} bath peptide concentration (Fig. 2).

This subfraction decreased the basal tone and amplitude of the contractions of the isolated rat duodenum, ileum and colon preparations as well as that of the electrically driven mouse vas deferens in the above dose range.

According to the paper chromatography this subfraction contained 4 ninhydrin-positive substances of which only one ($R_f = 0.08$) possessed inhibi-

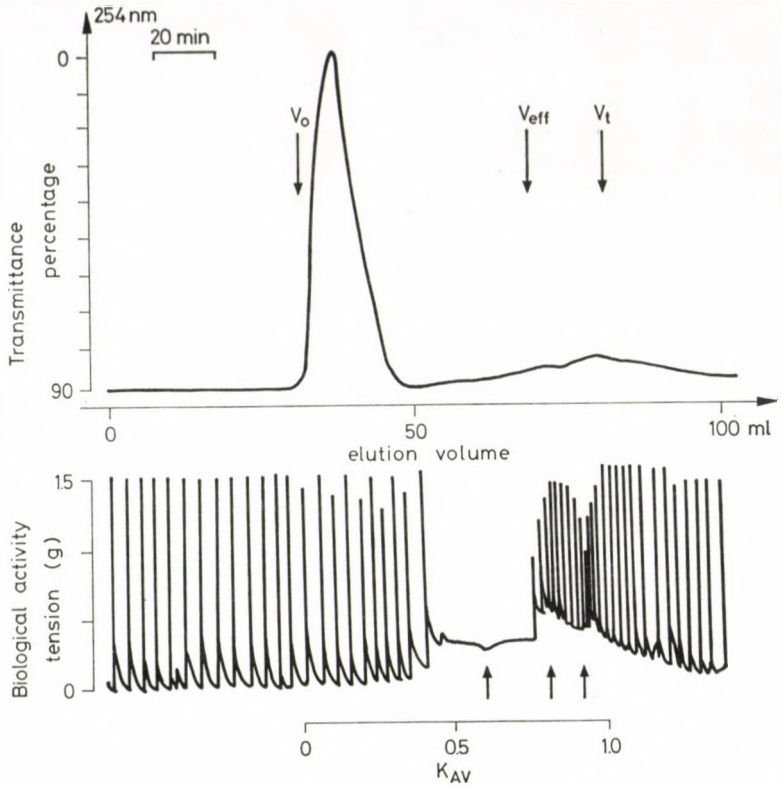

Fig. 1. Transmittance (upper part) and *in vitro* biological activity (lower part) were simultaneously recorded during the chromatography of 5.0 ml pooled human mid-pregnancy amniotic fluid on SG-25 gel column. Biological activity was detected on a (spontaneously contracting) pregnant rat uterine strip, immediately superfused with the eluted solution. Arrows under the motility recording indicate the biologically active fractions

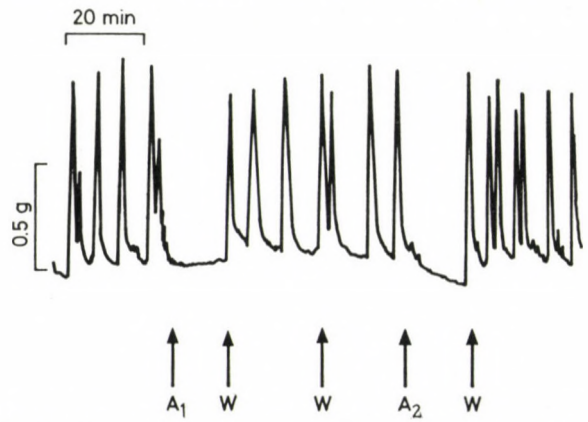

Fig. 2. Effect on the *in vitro* spontaneous contractions of human pregnant myometrial strip, of amniotic fluid fraction (*A*) that was separated by combining the SG-25 and CM-SC-25 chromatography. $A_1 = 3.6 \times 10^{-7}$ g ml^{-1} and $A_2 = 7.2 \times 10^{-7}$ g ml^{-1} peptide in the bath; W = wash out

tory effect on spontaneous contractions of the *in vitro* uterine strip of pregnant rat.

Discussion

Effect on uterine motility of the native amniotic fluid obtained in human mid-pregnancy has already been studied, the results are, however, contradictory [1, 8]. The present investigation indicates that the human amniotic fluid possesses both stimulatory and inhibitory effects on the *in vitro* spontaneous contractions of pregnant rat uterine strip.

According to the present knowledge, there are several factors in the body fluids involved in the regulation of contractility and motility of the myometrium in pregnancy. These factors have different chemical as well as physico-chemical and biological characters [2, 4]. In spite of the available data, the exact control of the mechanical function of the uterus during pregnancy has not satisfactorily been clarified. The present investigation has shown that amniotic fluid contains a factor, not identified so far that is capable of inhibiting the *in vitro* contractions of the pregnant rat and human myometrium. Its inhibitory effect is dose-dependent. On the basis of the described properties, this substance, partially purified seems to be non-specific and different from other endogenous substances with relaxing effect on the uterus (progesterone, relaxin, epinephrine etc.).

By the results presented above attention should be focused on that an endogenous factor not discovered so far might be involved in controlling the quiescence of the uterus in human pregnancy. This substance, called *uteroinhibin* belongs to the so-called middle molecular weight constituents of peptidic character, nominal molecular mass of which ranges between 500 and 5000 Dalton.

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THE FATE OF PANCREATIC SECRETORY PROTEINS IN THE BLOOD CIRCULATION OF DOGS AND RATS

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To follow the fate of intravascular pancreatic secretory proteins fresh pancreatic juice was collected from anaesthetized donor dogs and rats following stimulation of the prancreas by 4 U/kg b. w. of secretin + CCK-PZ. Protein level, lipase and amylase activity of the juice were determined, then the juice was injected into the blood stream of anaesthetized animals of the same species. Space of distribution, biological half-life, excretion in urine and bile, secretion in pancreatic juice and activity in thoracic duct lymph of the injected pancreatic secretory proteins were determined. Plasma volume of the recipient animals was also determined.

The space of distribution of both lipase and amylase and that of lipase in dogs was restricted to plasma volume. In dogs the biological half-life of amylase was longer than that of lipase. The excretion of enzymes in the bile of dogs and rats was found negligible, while in the urine of rats it reached 10 per cent of the injected amount. Pancreatic juice injected into the blood stream increased both lipase and amylase activities in the pancreatic juice of dogs, and the protein level and output in the pancreatic juice of both dogs and rats. A part of the enzymes which had left blood circulation is recirculated by the thoracic duct lymph into the blood.

An unresolved problem of pancreatic physiology is what happens with the pancreatic secretory proteins after they have entered the blood circulation: are they excreted from the blood or get into the pancreas and influence exocrine pancreatic secretion?

The aim of our study is to follow the fate of pancreatic secretory proteins in the blood by determining their biological half-life, space of distribution, excretion rate and their probable reuse [9] by the gland. Finally, the recirculation of pancreatic enzymes which had left the blood circulation was examined in thoracic duct lymph.

On the basis of data an attempt was made to establish how long can the increase of digestive enzyme activity be demonstrated in the blood plasma in acute pancreatitis, further, are the pancreatic digestive enzymes protected by some conservation mechanism [5] in favour of their reuse by the organism.

Materials and Methods

Group 1. Pancreatic juice was collected from 5 male donor dogs anaesthetized with chloralose and 36 male CFY donor rats anaesthetized with nembutal. Exocrine pancreatic function was stimulated by 4 U/kg b.w. of secretin + CCK-PZ (GIH Lab., Stockholm) given into the blood stream. The activity of lipase and amylase, and the protein level in the juice were determined. The juice was injected as a bolus into the femoral vein of animals of the same

species, i.e. into the blood of 6 recipient dogs (13.5 ± 0.8 kg b.w.) (mean ± SEM) and of 12 recipient rats (295 ± 11 g b.w.). The recipient animals were anaesthetized in the same way as the donors. After the intravenous injection of pancreatic juice arterial blood samples were taken in consecutive periods for 90 min. The experimental design is shown in Fig. 1. Excretion of both enzymes was measured in the urine and from the cannulated common bile duct of recipient dogs during this period. Lipase activity was measured by the method of WEBER [10] using sunflower oil as substrate, amylase activity was measured by the method of BERNFIELD

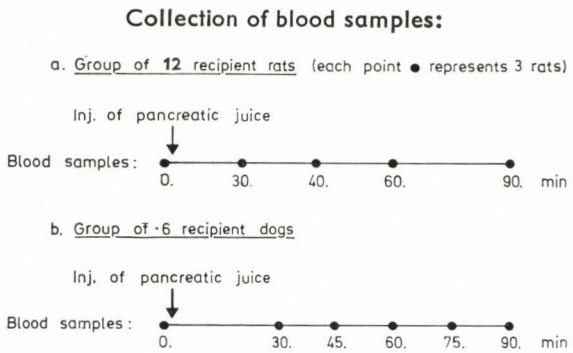


Fig. 1. Experimental design of Group 1

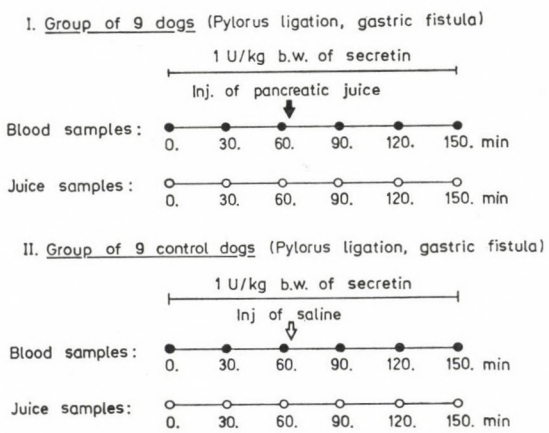


Fig. 2. Experimental design of Group 2

[2] using Merck starch as substrate. The enzyme activities measured in plasma samples were extrapolated to 0 min. The space of distribution of lipase and amylase was determined by using the formula:

$$\text{space of enzyme distribution} = \frac{\text{injected amount of enzyme} - \text{excreted amount of enzyme}}{\text{enzyme activity extrapolated to 0 min} - \text{basal enzyme activity in plasma}}$$

In both species plasma volume was determined by intravenously injected 1% Evans blue solution.

Group 2. Pancreatic juice was collected from donor male dogs anaesthetized by chloralose and from donor rats anaesthetized by urethane by stimulating the exocrine pancreatic secretion with intravenous 4 U/kg b.w. of secretin + CCK-PZ (GIH Lab. Stockholm). The collected juice was weighed, lipase and amlyase activity and protein level [11] were determined. 18 recipient dogs were laparotomized, the pylorus was ligated and a gastric fistula was prepared. A polyethylene cannula was introduced into Santorini's duct and the small pancreatic duct was ligated. Pancreatic juice was collected for 150 min in 30 min periods by stimulating the exocrine pancreatic secretion with 1 U/kg b.w. of secretin (GIH Lab., Stockholm) given into the femoral vein for 150 min with a Harvard infusion pump. Arterial blood samples were taken at 0 min and subsequently every 30 min. After the second collection period of the juice — that is in the 61st min — 2.8 ± 0.7 ml/kg b.w. fresh pancreatic juice was given intravenously to 9 dogs (18 ± 1 kg b.w.) as a bolus. Further 9 dogs (18 ± 2 kg b.w.) served as control receiving physiological saline instead of pancreatic juice. The experimental design is shown in Fig. 2.

Twenty recipient male CFY rats (300 g b.w.) were subjected to laparotomy. The common bile duct was ligated at the hepatic hilum and a polyethylene cannula was introduced into the duct close to the duodenum for collecting pancreatic juice. Ten of the rats received fresh pancreatic juice (1 ml/kg b.w. = 48 mg protein/kg b.w.) at 0 min as a bolus into the femoral vein. Ten untreated rats served as controls. Pancreatic juice was collected for 60 min. Each rat was exsanguinated from the abdominal aorta in the 60th min. Volume, lipase [10], amylase [2] activity and protein [11] level of pancreatic juice samples of recipient dogs, furthermore, lipase and amylase activity in their plasma were measured. Volume, amylase activity and protein level were measured in the pancreatic juice of donor and recipient rats, and the activity of amylase was measured in the plasma of recipient rats.

Group 3. From male CFY donor rats pancreatic juice was collected during stimulation of exocrine pancreatic secretion by 4 U/kg b.w. of secretin + CCK-PZ (GIH Lab., Stockholm) given into the femoral vein. Volume of the juice, lipase and amylase activity and the protein level were measured. The pancreatic juice was injected into the femoral vein of 30 male CFY recipient rats. Five groups were composed from these rats each consisting of 6 rats. The thoracic duct lymph was collected for 30, 45, 60, 90 and 120 min periods and after the collection had been finished the rats were exsanguinated from the abdominal aorta. Another group (control) consisting of 6 rats did not receive pancreatic juice. In a further group consisting also of 6 rats the common bile duct was ligated, the pancreatic function was stimulated by 4 U/kg b.w. of secretin + CCK-PZ (GIH Lab., Stockholm) and thoracic duct lymph was collected for 150 min. The rate of the latter two groups were also exsanguinated from the abdominal aorta.

In each of these groups plasma samples or plasma and lymph samples were analyzed for lipase [10] and amylase [2] activity.

The mathematical-statistical analysis of groups 1–3 was performed by Student's *t*-test or after analysis of variance by DUNNETT [4] and DUNN [3] contrasts.

Results

Group 1. The disappearance of pancreatic lipase and amylase from the blood stream of dogs is shown in Fig. 3. In dogs the space of lipase nearly corresponded to the plasma volume (5% of b.w.), that of amylase being about twice of it (10%) ($p < 0.01$). The biological half-life of lipase (80 min) was shorter ($p < 0.01$) than that of amylase (434 min) (Table I).

The disappearance of pancreatic lipase and amylase from the blood circulation of rats is shown in Fig. 4. In rats the space of both lipase and amylase nearly corresponded to the plasma volume (4% of b.w.).

The biological half-life of lipase (72 min), similarly as in dogs, was also shorter than that of amylase (87 min) (Table II). When expressed in per cent of the injected enzyme amount, lipase and amylase excretion was negligible in the urine of dogs and rats, while excreted enzyme activity rose to about 10% in urine of rats during 90 min (Table III).

Table I

Plasma volume, space of distribution and biological half-life of lipase and amylase in dogs

	Body weight (b.w.) kg	Plasma volume		Volume of distribution of				t 1/2, min		Injected total activity of	
				lipase		amylase					
		litre	% b.w.	litre	% b.w.	litre	% b.w.	lipase	amylase	lipase I.U.	amylase S.U.
No. of dogs	5	5	5	5	5	5	5	5	5	5	5
Mean	13.5	0.73	5.40	0.84	6.2	1.35*	10.1*	79.6	434.0	4651.5	60 437.7
± SEM	0.8	0.06	0.26	0.13	1.0	0.12	0.9	6.7	81.2		

$p < 0.01$

I.U. = International Unit
S.U. = Somogyi Unit
*$p < 0.01$ vs $\left\langle \begin{matrix} \text{plasma volume} \\ \text{vol. of distrib. of lipase} \end{matrix} \right.$

Table II

Plasma volume, space of distribution and biological half-life of lipase and amylase in rats

	Body weight (b.w.) g	Plasma volume		Body weight (b.w.) g	Volume of distribution of				t 1/2, min		Injected total activity of	
					lipase		amylase					
		ml	% b.w.		ml	% b.w.	ml	% b.w.	lipase	amylase	lipase I.U.	amylase S.U.
No. of rats	15	15	15	18	3	3	3	3	3	3	3	3
Mean	313.3	12.9	4.3	286.0	13.1	4.6	11.8	4.1	72.2	86.5	39.7	658.6
±SEM	18.1	0.6	0.3	—	—	—	—	—	—	—	—	—

I.U. = International Unit
S.U. = Somogyi Unit

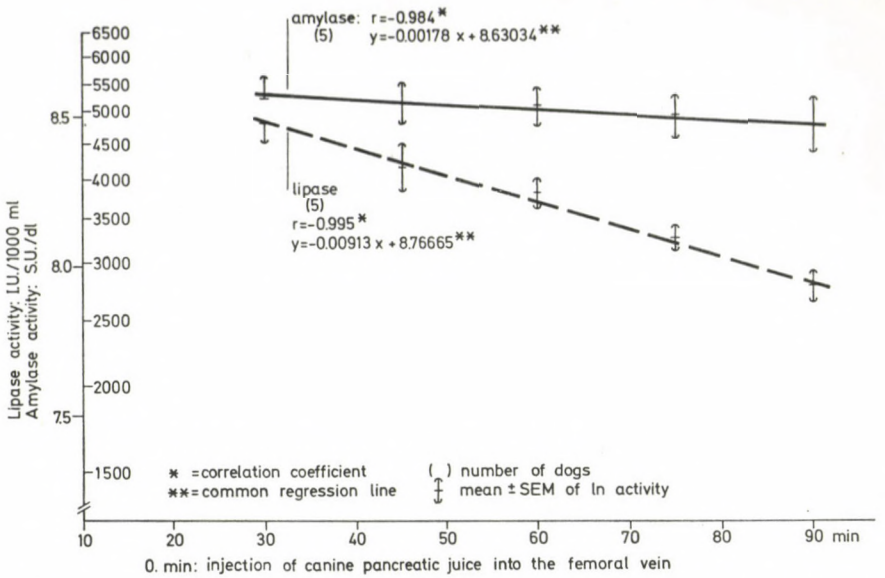


Fig. 3. Disappearance of pancreatic lipase and amylase from the blood circulation of dogs

Group 2. In recipient dogs injection of pancreatic juice raised lipase and amylase activity in blood plasma, while not affecting the volume of pancreatic juice (Fig. 5), or enzyme output in the juice (Figs 6, 7), however, the injection of juice induced a tendency to increase both lipase (Fig. 8) and amylase (Fig. 9) activity in the juice. It increased both the protein level and output in juice (Figs 10–11) of recipient dogs as compared to the corresponding control values.

Table III

Excretion of lipase and amlyase in urine and bile of dogs and rats after intravenous injection of homologous pancreatic juice

	Excreted lipase activity I.U.		Excreted amylase activity S.U.		Injected total activity	
	urine	bile	urine	bile	lipase I.U.	amylase S.U.
In dogs	65.4 (7)	6.1 (7)	1019 (7)	108.4 (7)	5567 (7)	8498 (7)
% of the injected activity	0.9	0.2	1.3	0.2	100	100
In rats	2.5 (5)	0.1(5)	66.8 (5)	2.1 (5)	22.6 (5)	506.5 (5)
% of the injected activity	11	0.5	13	0.4	100	100

Number of animals in brackets

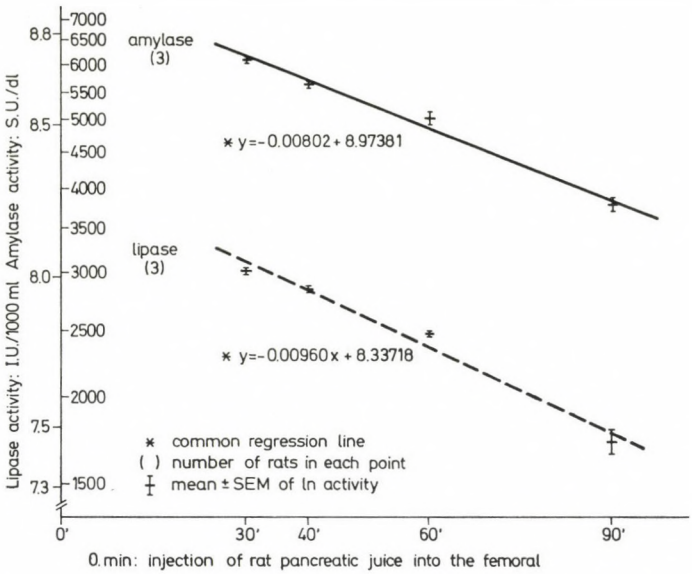


Fig. 4. **Disappearance of lipase and amylase from the blood circulation of rats**

In recipient rats injection of pancreatic juice did not affect the volume, the enzyme activity and output of pancreatic juice, while increasing the protein level (control, 14.6 g/l protein; injected group, 20 g/l protein) ($p < 0.05$) and output (control, 1 mg/60 min; injected group, 1.5 mg/60 min) ($p < 0.05$).

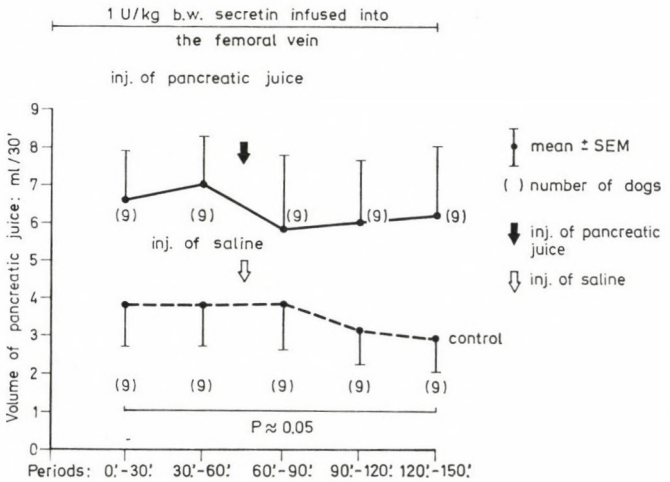


Fig. 5. **Volume of pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice**

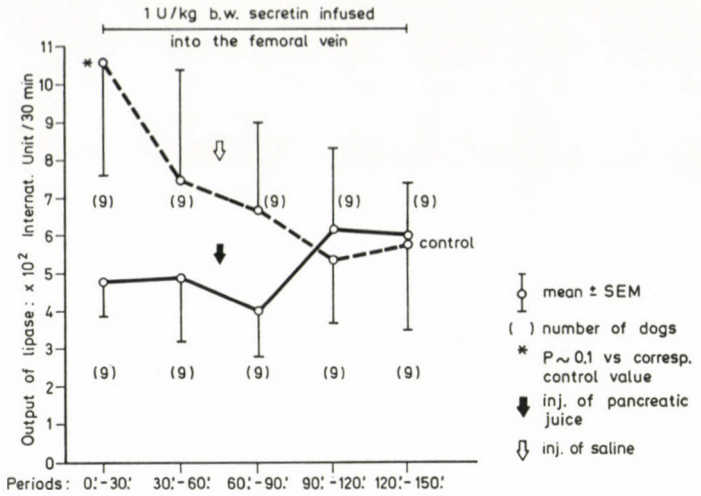


Fig. 6. Lipase output in pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

Due to the injection of pancreatic juice, activity of lipase and amylase increased in plasma and lymph; arterial lipase and amylase activity started to decrease from the 30th min after the injection, the activity of enzymes in lymph remained constant from the 45th min after the injection (Figs 12, 13).

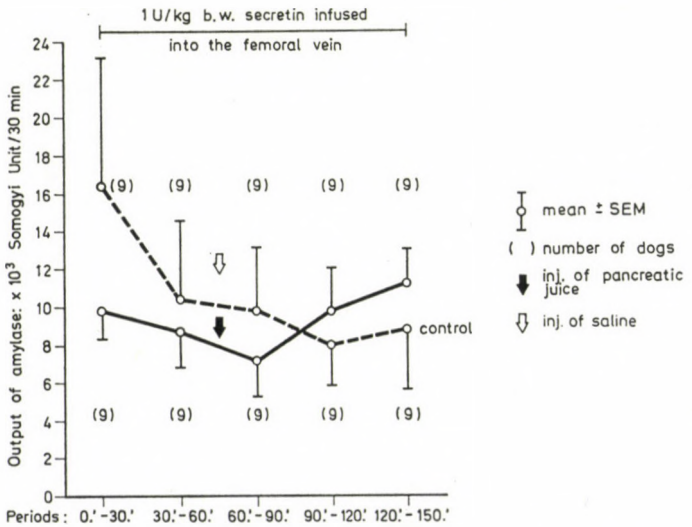


Fig. 7. Amlyase output in pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

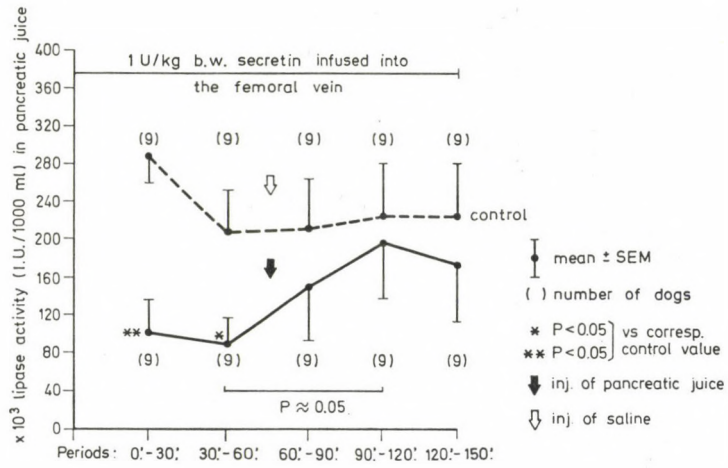


Fig. 8. Lipase activity of pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

In the 150th min, in rats the pancreatic duct of which had been ligated and the exocrine pancreas was stimulated simultaneously, the activity of enzymes in blood plasma and lymph was about the same as in rats into which pancreatic juice had been injected.

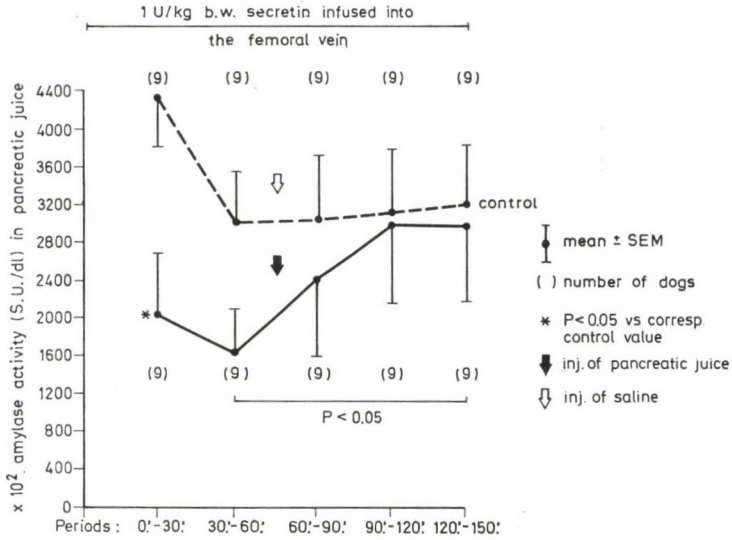


Fig. 9. Amylase activity of pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

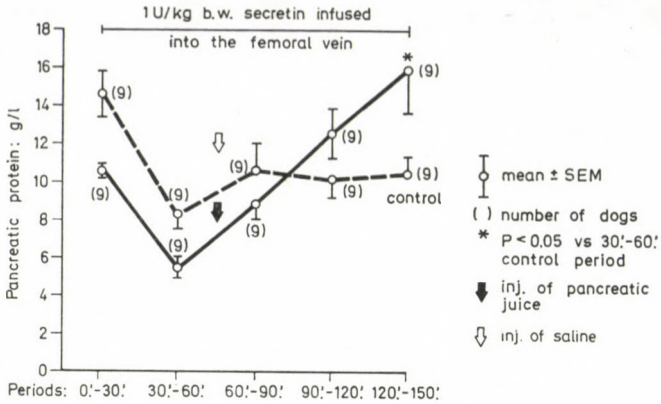
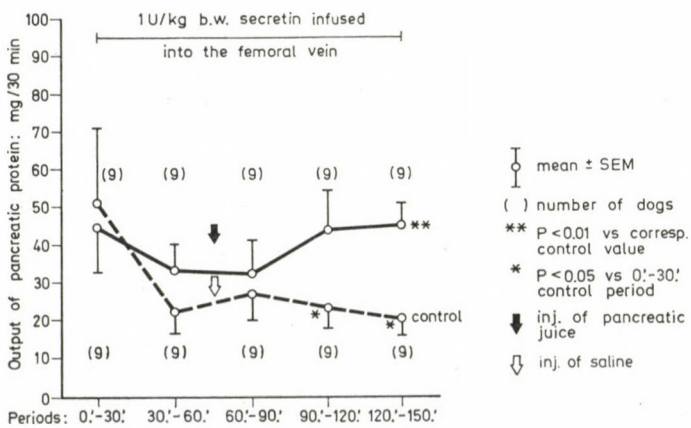


Fig. 10. Protein level of pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

Discussion

It is surprising that in dogs and rats the biological half-life of amylase is longer than that of lipase while in humans this is regarded as reversed. It should be mentioned that lipase and amylase in pancreatic juice of dogs and rats did not change *in vitro* when incubated with homologous plasma or saline at 37 °C for 90 min. Thus, digestive enzyme activity in blood plasma of these species is not influenced by other components (e.g. enzymes) of the plasma (Papp, unpublished data). The fact that the lipase-space corresponds



Gig. 11. Protein output of pancreatic juice of recipient dogs after intravenous injection of canine pancreatic juice

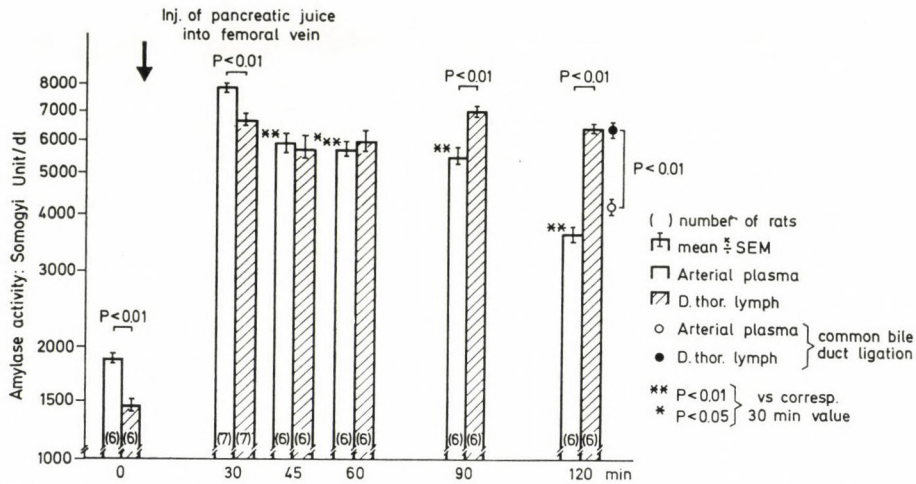


Fig. 12. Lipase activity of thoracic duct lymph and arterial blood plasma of rats after intravenous injection of rat pancreatic juice

to plasma volume indicates that lipase in the blood is bound to plasma proteins. The biological half-life of labelled rat pancreatic proteins was found to be 17 min [8] which is shorter than that of lipase and amylase in rats. APPERT et al. found that the biological half-life of both lipase and amylase was 3 h in dogs [1]. Their findings and our data cannot be compared, however, since these authors did not publish the enzyme activity extrapolated to 0 min and the disappearance rate of the enzymes from the blood.

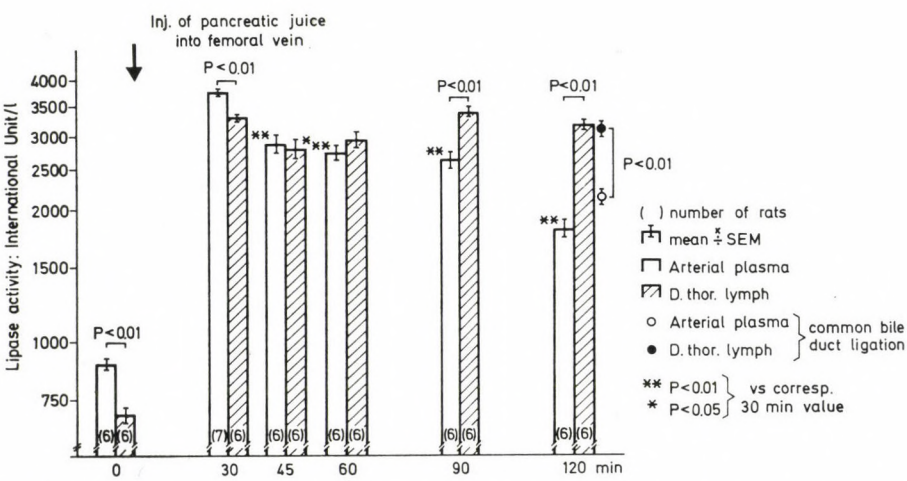


Fig. 13. Amylase activity of thoracic duct lymph and arterial blood plasma of rats after intravenous injection of rat pancreatic juice

The mechanism of the stimulating effect of intravascular pancreatic proteins on the protein secretion in pancreatic juice has remained uncleared. It is not known whether the intravascular pancreatic proteins are excreted by the pancreas, or the intravascular proteins stimulate the secretion of the pooled intra-acinar secretory proteins. It is beyond doubt that this phenomenon occurs only after a delay. Consequently, it is not due to certain peptides which may have been secreted into the juice. From a biological point of view this mechanism does not seem to be very effective: in rats calculation revealed that about 3% of the injected secretory proteins were secreted into the juice by the pancreas during the 60 min period following the injection [9]. The flux of pancreatic proteins into the acinar cells may take place accross basolateral membranes from the interstitium [6, 7]. Clearly, the best method to elucidate the mechanism of secretion of intravascular pancreatic proteins into the juice would be the use of radioactive labelled pancreatic enzymes and to follow their pathway into the acinar cells and afterwards into the secretion. So far a sufficient amount of these enzymes is not available, thus our results presented in this paper should be regarded as a convincing hypothesis as to the re-use of the intravascular pancreatic proteins by the organism.

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EFFECT OF MAZINDOL ON FEEDING BEHAVIOUR AND ON NORADRENERGIC FUNCTION OF VARIOUS PARTS OF THE RAT BRAIN

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The effect of mazindol on feeding behaviour was investigated in rats fasted for 24 hours. They were then given mazindol i.p. and placed in a new environment. The effect of the drug on the disappearance of ^{3}H-noradrenaline (^{3}H-NA) from various parts of the brain after injection into the cerebral ventricles was also investigated. Mazindol was more potent in suppressing feeding behaviour than in reducing food intake, indicating that a number of aspects of feeding behaviour may be more sensitive indices of the anorectic effect of a drug than food intake per se. Mazindol, in a dose of 30 mg/kg, accelerated the disappearance of ^{3}H-NA from the medial hypothalamus but not from other areas of the brain, suggesting that the drug has a locus-specific action on cerebral noradrenaline metabolism.

Mazindol (5-p-chlorophenyl-2,3-dihydro-5H-imidazo [2,1-a] isoindol--5-ol) is a potent anorectic [7] which has been used successfully in the treatment of obesity. An interesting component of the action of mazindol is its effect on extrahypothalamic structures. GOGERTY et al. [7] found that lesion of the pellucid septum reduced the suppressant effect of mazindol on mouse-killing behaviour in rats. The view that behavioural adaptation to stress and feeding behaviour are intimately related is based on the observation that stress induces overfeeding in rats [2, 16] and possibly also in man [12, 20]. These findings indicate the importance of extrahypothalamic structures in the regulation of feeding behaviour, and it is possible that the beneficial effect of mazindol is partly due to these extrahypothalamic effects. In order to test this hypothesis, we investigated the effect of mazindol on the feeding behaviour of fasted rats in a new environment, i.e. in a stressful situation, which activates the brain structures involved in the behavioural response to stress under such circumstances, food-seeking behaviour motivated by hunger conflicts with exploratory activity evoked by external stimuli.

Pursuing a different line of investigation, other authors have shown that mazindol alters the function of monoaminergic neurones, which are thought to be closely involved in the regulation of feeding behaviour and food intake. Mazindol is a potent inhibitor of noradrenaline (NA) uptake *in vitro*, and also inhibits dopamine and serotonin uptake [10]. Likewise it inhibits NA uptake *in vivo* [5, 17, 21]. It has also been established that NA inhibits food intake in rats [1, 8]. In the present study, experiments were

carried out to investigate the effect of mazindol on the disappearance from the medial and lateral hypothalami and from various extrahypothalamic structures of ^{3}H-NA injected into the cerebral ventricles.

Methods

Laboratory animals and mazindol administration

Male rats of a Wistar-derived inbred strain, 180–200 g body weight were used. They were starved for 24 h before the behavioural test, but water was provided ad libitum. One hour before the behavioural test 0.625–5.0 mg/kg body weight mazindol* and for the neurochemical studies, doses of 10–30 mg/kg were injected i.p. of mazindol solution prepared by dissolving the compound in 0.5 N HCl, neutralising the solution and diluting it with physiological saline. Control animals were given injections of the solvent. After administration of mazindol the rats were returned to their usual cage and observed for gross behavioural effects, i.e. stereotyped movements.

Behavioural test

After a fast of 24 h, the rats were put in separate boxes measuring $30 \times 30 \times 30$ cm, with a transparent front wall, through which they were illuminated. The base of each box was divided into 4 squares. A dish of food pellets (Gödöllő, Hungary) was placed in the middle of each box. The animals were observed for 10 minutes from the moment they were put into the box, and the following variables were recorded:

1. feeding latency: the time (in s) elapsing until animals had begun to feed, i.e. picking up a piece of food and chewing it
2. feeding time (in s)
3. exploration: the number of times the animal crossed the lines, the number of rearings and the time the animal spent in the upright position were noted and added to give a combined score.

Food intake in g/100 g body weight was measured at the end of the 10 min observation period and again after 60 min. In another experiment the behavioural excitatory action of mazindol was tested by measuring exploration in the experimental box described above. In this experiment the rats were not deprived of food and were considered satiated. Furthermore, no food was present during the testing exploratory activity.

^{3}H-noradrenaline administration

An indwelling polyethylene cannula (inside diameter 0.35 mm, outside diameter 0.8 mm) was implanted into a lateral ventricle of the brain under ether anaesthesia 4 days before the injection of ^{3}H-NA. The stereotaxic coordinates of the tip of the cannula, according to De Groot's Atlas [4] were as follows: A 5.8, L 1.3, D 2.8. On the test day 1 μCi of ^{3}H-NA (1-, 7-^{3}H-noradrenaline, 5.8 Ci/mmol, Amersham) in a volume of 30 μl was injected into the cerebral ventricle. The NA was dissolved in physiological saline containing 0.1% (w/v) ascorbic acid as an antioxidant and adjusted to pH 7.0 immediately before administration. One hour after the injection of ^{3}H-NA, mazindol was injected i.p. in doses of 10 or 30 mg/kg body weight. The animals were decapitated 4 h after the injection of mazindol. The brain was removed at once and cooled on ice while the following structures were dissected out: (i) the medial hypothalamus including the median eminence and the arcuate, ventromedial and dorsomedial nuclei; (ii) the lateral hypothalamus adjacent to the anterior and medial hypothalamus; (iii) the hippocampus; (iv) the locus coeruleus and (v) the remaining parts of the pons and the medulla oblonga. The locus coeruleus was dissected out from the pons as described by Segal and Kuczenski [18]. The ^{3}H-NA content of these regions of the brain was extracted by the aluminium absorption technique and estimated by liquid scintillation counting as described earlier [3].

* Kindly supplied by Sandoz Ltd., Basel, Switzerland.

Results

Effect of mazindol on feeding behaviour

Changes in feeding latency, the time spent feeding, exploratory activity and food intake under the effect of increasing doses of mazindol are shown in Fig. 1. Even the lowest dose (0.625 mg/kg) of mazindol produced a signif-

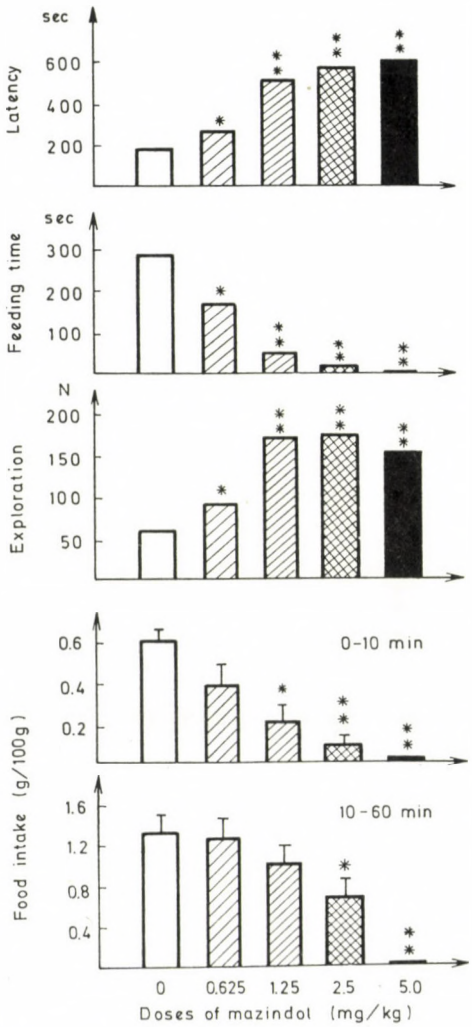

Fig. 1. Effect of mazindol on latency of initiation of feeding, on feeding time and exploration (in medians), and on food intake (mean ± S.E.M.) of 24 h fasted rats subjected to a novel environment. Food intake was measured during the first 10 min behavioural observation period and in the subsequent 50 min period. The number of animals varied from 6 to 8 per group. $p < 0.05$, $p < 0.01$

icant ($p < 0.05$, Mann-Whitney test) change in all behavioural variables. The increase in latency, decrease in feeding time and increase in exploratory behaviour were more pronounced after 1.25 and 2.5 mg/kg doses. A dose of 5 mg/kg abolished feeding completely and induced stereotyped movements such as increased locomotor activity, repetitive rearings and head turnings with sniffing during the postinjection period and behavioural test. The threshold dose causing a change in food intake was higher than that causing a change in feeding behaviour. The lowest doses reducing food consumption in the first 10 min and in the subsequent 50 min period were 1.25 mg/kg and

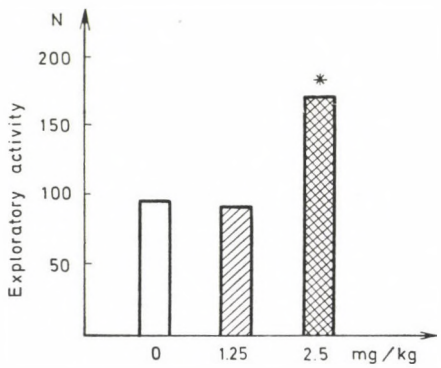

Fig. 2. Effect of mazindol on exploratory activity of satiated rats. Number and duration of rearings and number of crossings were scored (N). Six rats were used in each group. $p < 0.05$

2.5 mg/kg, respectively. Exploratory activity was also measured in the satiated rats after injection of the drug. The lowest dose causing an increment in exploratory activity under these conditions was 2.5 mg/kg (Fig. 2).

Table I

Effect of mazindol on the disappearance of ^{3}H-noradrenaline (NA) in several brain areas

Groups	^{3}H-NE content in μCi/g tissue				
	Medial hyopthalamus	Lateral hypothalamus	Locus coeruleus	Hippocampus	Medulla and pons
Saline	199 ± 23	35.4 ± 3.1	33.4 ± 3.3	18.8 ± 2.2	20.9 ± 2.8
Mazindol 10 mg/kg	170 ± 16	28.3 ± 4.2	34.4 ± 2.6	22.9 ± 3.7	27.6 ± 3.4
Mazindol 30 mg/kg	108 ± 10[a]	38.3 ± 4.3	33.6 ± 3.0	21.7 ± 2.8	22.6 ± 3.1

Values are means ±S.E.M.
6 rats per group were used
a = $p < 0.01$ vs. saline control group (Student's t test)
Mazindol was injected 1 h after the intraventricular injection of 1 μCi ^{3}H-NA
Decapitation occurred 4 hours after the mazindol injection

Effect of mazindol on brain noradrenergic function

The effect of mazindol on disappearance of ^{3}H-NA from various parts of the brain was investigated. Labelled NA and one hour later mazindol was injected into the cerebral ventricle. Four hours later the concentration of ^{3}H-NA was unchanged (Table I) in all regions except the medial hypothalamus. Disappearance of ^{3}H-NA from the medial hypothalamus was increased only by 30 mg/kg of mazindol and this resulted in a lower ^{3}H-NA tissue concentration.

Discussion

When rats are placed in a new environment, external stimuli evoke intense exploration which normally inhibits certain types of spontaneous behaviour including feeding. Thus a model of this type would be expected to provide a sensitive index of anorectic efficacy. The tests carried with mazindol have confirmed this assumption. In a dose as low as 0.6 mg/kg, mazindol increased the feeding latency, reduced time spent feeding and increased locomotor activity in rats which had been fasted for 24 hours. The increase in exploration was accounted for by the reduced feeding time, since in satiated rats an increase in exploratory activity was observed only after a mazindol dose of 2.5 mg/kg. Our findings confirm those of GOGERTY et al. [7] indicating that, up to a certain dose level, mazindol exerts its anorectic effect without increasing locomotor activity or stimulating behavioural responses to a new environment. The variables related to feeding behaviour mentioned above, i.e. feeding latency, time spent feeding and exploratory activity, proved to be more sensitive indices of the appetite-suppressing effect of mazindol than food intake whether measured in the initial 10 min or over a longer 50 min observation period. Low doses of mazindol, with marked effects on feeding behaviour, did not reduce food intake. The inhibitory role of the medial and the facilitatory role of the lateral hypothalamic structures in the regulation of food intake is well documented (for review see GROSSMAN [9]). It is also known that some extrahypothalamic structures such as parts of the forebrain-limbic midbrain system [14] affect food intake. Among these structures the septal area is involved in the regulation of food intake [13, 15, 19].

Mazindol is known to affect the septum, since lesions in this area abolish the suppressive effect of mazindol on mouse-killing behaviour [7]. Since the septum is of crucial importance in behavioural adaptation to stress, and since stress may, under certain circumstances, increase food intake [2, 16] the effect of mazindol on this area of the brain merits further investigation to establish its precise mechanism of action. The ventral noradrenergic bundle (VNAB) inhibits food intake, an effect which is probably mediated by the

lateral hypothalamus [1, 8, 11]. Hyperphagia induced by lesion of the ventromedial hypothalamus is associated with a decrease in NA turnover in the lateral hypothalamus [3]. Although hyperphagia induced in this way is due to a number of factors besides the decreased noradrenergic function [11], it is possible that the impairment of noradrenergic function in the hypothalamus may have some role to play in the development of hypothalamic obesity. Anorectic agents such as mazindol, amphetamine and other phenyl-ethylamine derivatives affect cerebral NA metabolism [6]. The anorectic effect of amphetamine is weakened by lesions of the VNAB [1]. Mazindol is a potent inhibitor of NA uptake through the presynaptic membrane [5, 10, 17, 21], an effect which is probably involved in its anorectic action. After the administration of 30 mg/kg mazindol i.p. we found that ^{3}H-NA disappearance was faster in the medial hypothalamus than in other areas of the brain investigated, i.e. the lateral hypothalamus, locus coeruleus, pons, medulla oblongata and hippocampus. In similar experiments a dose of 25 mg/kg mazindol administered orally did not affect the disappearance and metabolism of ^{3}H-NA however, in these oral studies the whole brain was analysed [5]. Thus it appears that mazindol might have a locus-specific effect on cerebral NA metabolism by its effect on the medial hypothalamus. The medial hypothalamus has a monosynaptic connection with the amygdaloid nucleus through the stria terminalis, but is connected oligosynaptically with all the extrahypothalamic structures involved in the regulation of food intake [14]. Whether the effect of mazindol on NA metabolism in the medial hypothalamus was a direct one or a result of the drug's extrahypothalamic action is not known. It should be borne in mind that mazindol affects cerebral noradrenergic function only in massive doses, hence this effect is not a primary component of the anorectic effect of mazindol.

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PARTIAL PURIFICATION FROM BOVINE PULMONARY TISSUE OF A PROTEIN CAPABLE OF INHIBITING *IN VIVO* DNA SYNTHESIS IN MOUSE PULMONARY CELLS

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This paper reports on partial purification and characterization of a natural (endogenous) factor capable of inhibiting *in vivo* DNA synthesis in mouse pneumocytes in a tissue-specific manner. By using a combination of ultrafiltration and various chromatographic techniques, the active agent has been partially purified from aqueous extracts of both bovine and rat pulmonary tissue. The factor responsible for the observed effect was found to be a heat labile compound, most likely a protein of approx. 40 000 molecular weight. Its chemical and physicochemical properties determined so far, and also the manner of its biological action implies that this lung tissue derived agent might be an endogenous proliferation inhibitor with a chalone-like character operating in the pulmonary epithelial cells .

The experiences accumulated during the past few years have favoured the idea that specific endogenous substances capable of stimulating [1, 2] and inhibiting [3, 4] *in vivo* and *in vitro* cell proliferation might play a decisive role in the control of tissue mitotic homeostasis in mammalian organisms. Their theoretical significance and the chance of their clinical applicability [5, 6, 7] lends a particular significance to a much debated and still controversial group of endogenous substances, called chalones, with the assumed capability of inhibiting cell proliferation in a tissue or cell-line specific manner [8, 9, 10, 11].

As to the endogenous proliferation inhibitors of the pulmonary cells, it was SIMNETT and co-workers [18] who first demonstrated in short-term lung explants the tissue-specific proliferation inhibitor capacity of crude aqueous extracts of bovine pulmonary tissue. A few years later HOUCK [19] showed that 30 000–50 000 Dalton ultrafiltrates prepared from an acetone dry powder of bovine pulmonary tissue, cell-line specifically inhibited the *in vitro* proliferation of cultured bronchial cancer cells.

In the present report we describe a factor probably of protein character that has partially been purified from the aqueous extract of bovine pulmonary tissue, which tissue-, and seemingly also cell-line specifically inhibits *in vivo* DNA synthesis in some of the mouse pulmonary cells, first of all in type 2 pneumocytes.

Methods

Preparation of tissue extract

The pulmonary tissue (ordinarily 4 kg), freshly removed from slaughtered cattle was placed in a pre-cooled (4 °C) container and transferred quickly into the laboratory. On each occasion, processing of the tissue was started under carefully cooled conditions (2–4 °C) within 40 min following slaughter of the animals.

In order to increase its effectivity, tissue extraction was carried out in several steps. After rough chopping, the tissue was washed in ice cold normal saline solution and ground twice with an electric grinder placed in a refrigerator. The ground tissue was suspended in a tenfold volume of ice cold distilled water followed by 2 min mincing in 1500 ml portions by means of a waring blender (20 000 rpm). The suspension was thereafter sonicated (output = 70 W) in a refrigerator for 10×2 min and allowed to stand overnight at 2 °C.

Next morning, the suspension was filtered through 4 layers of gauze followed by addition of a tris-HCl buffer (pH 5.5) in 0.1 mol/l final concentration. The buffered filtrate was sedimented at 2 °C for 30 min in the zonal rotor of a Beckman J 21 B type centrifuge. The resulting supernatant was used for fractionation.

Fractionation of the extract

a) *Ultrafiltration:* the suspension obtained as previously described was filtered through a capillary membrane filtration unit (Amicon H1P100; nominal cut-off limit, 100 000 Daltons) operated as a recirculation system. In order to ensure extensive filtration, the residue was exhaustively filtered through the same system by continuous addition of ten volumes of a tris-HCl buffer (0.1 mol/l; pH 5.5). The filtrate was concentrated on a capillary membrane filtration unit (Amicon H1P10; nominal cut-off limit, 10 000 Daltons) operated as a periodic system. The hydrogen ion concentration of the retained material was occasionally adjusted to pH 8 by extensive dialysis against a tris-HCl buffer solution (0.1 mol/l; pH 8). The use of the two capillary membrane filtration units with different cut-off limits as specified above allowed to obtain a final retentate containing solutes with nominally less than 100 000 but more than 10 000 Dalton molecular mass.

b) *Column chromatography:* the retentate prepared as described in the previous paragraph was chromatographed on a DEAE Sephadex A-50 gel column (5×85 cm) by using the fractional gradient elution technique. Elution was started with a tris-HCl buffer (0.1 mol/l, pH 5.5) and proceeded, when the first peak appeared, with a NaCl gradient at a flow rate of 0.65 ml/min. Elution buffers with the following composition were applied: (a) 1100 ml tris-HCl (pH 8.0); (b) 900 ml tris-HCl containing NaCl in a concentration of 0.05 mol/l, (pH 7.3); (c) 1100 ml tris-HCl containing NaCl in a concentration of 0.1 mol/l (pH 7.0); (d) 900 ml tris containing NaCl in a concentration of 0.35 mol/l (pH 6.0). The optical activity of the eluate was monitored continuously at 280 nm. The eluted fluid was pooled in four fractions and lyophilized.

The only DEAE-Sephadex fraction that disclosed biological activity was further purified on a CM Sephadex G-25 column (2.5×100 cm) previously equilibrated with a tris-HCl buffer (0.1 mol/l, pH 5.5). Fractional elution was carried out with buffers of different composition in which, besides NaCl of specified concentration, tris was also included in 0.1 mol/l final concentration. The pH of the latter was occasionally adjusted to the desired value with acetic acid. Elution at a flow rate of 0.8 ml/min was undertaken with the following solutions: (a) 300 ml tris-acetate (pH 5.5); (b) 250 ml NaCl (0.025 mol/l, pH 5.7); (c) 250 ml NaCl (0.05 mol/l, pH 6.0); (d) 250 ml NaCl (0.075 mol/l, pH 6.25); (e) 300 ml NaCl (0.1 mol/l, pH 6.5); (f) 500 ml NaCl (0.3 mol/l, pH 7.5). Optical activity at 280 nm was continuously recorded. The eluate was pooled in four fractions followed by lyophilization.

The fraction disclosing biological activity was further purified on a Sephadex G-75 column (2.6×200 cm) previously equilibrated with PBS. Elution was done in this case with PBS at a flow rate of 0.44 ml/min and the 280 nm optical activity was monitored continuously. The column was calibrated with albumin fraction V ($Mw = 6.5 \times 10^4$) and cytochrome C ($Mw = 1.26 \times 10^4$).

Molecular weight determination

Determination of the approximate molecular weight of solutes included in the only biologically active CM Sephadex G-25 fraction was carried out on a Sephadex G-75 column prepared and calibrated as described above, as well as by ultracentrifugation by using the sedimentation rate technique referred to an albumin standard [14].

Chemical analysis

Protein was determined with LOWRY's method by using bovine albumin as standard [16]. DNA content was assessed by BURTON's procedure [6].

Measurement of biological activity

In order to ensure removal of undesired low molecular weight components from the eluates, all chromatographic fractions were extensively dialyzed against PBS prior to testing of their biological activity.

1. *In vivo screening test.* Biological activity of DEAE Sephadex fractions, which were regarded as only slightly purified, was tested on groups of animals consisting of 30 mice (25 ± 3 g) each, by intraperitoneal administration of lyophilized and redissolved chromatographic fractions containing identical amounts of protein. Single doses amounted to 12.5 mg protein/kg body weight, which were repeated four times at 1.5 h intervals. Thus, the full dose administered in 6 h amounted to 50 mg protein/kg body weight. In the 5th h after the first dose, ^{3}H-TdR was administered intraperitoneally (0.5 mCi/kg body weight). The controls were treated with PBS under identical conditions. The mice were decapitated 1.5 h after the administration of the label. Identical pulmonary lobes were removed from each animal, their weight assessed with 0.5 mg accuracy, and placed subsequently in scintillation vials containing 1 ml of a solubilizing agent (Soluene-100, Packard). In order to promote dissolution of the tissue, the vials were shaken overnight at room temperature with 150 min^{-1} frequency. Following complete solubilization 10 ml of a toluene-based scintillation fluid containing PPO-POPOP was pipetted into the vials. After allowing 4 h for standing, the radioactivity was determined by using a Packard 2110 liquid scintillation spectrometer. For assessing efficiency of individual measurements, determination of radioactivity was carried out by automatic external standardization. Results were expressed as dpm/mg tissue wet weight.

2. *In vivo measurement of ^{3}H-TdR incorporation, into DNA.* The *in vivo* biological activity and occasionally also tissue specificity of the more extensively purified CM Sephadex G-25 fractions was tested by assessing radioactivity incorporated into a unit quantitity of DNA isolated from the investigated tissues. The test was performed on groups of animals comprising 20 mice each. Test substances were administered as described in the previous paragraph with the only difference that, in these experiments, single doses amounted to 7.5 mg protein/kg body weight and thus, the full dose administered amounted to 30 mg protein/kg body weight. Control animals received PBS under identical conditions. After decapitation, two identical pulmonary lobes were removed and processed separately for parallel measurements. The removed lobes were homogenized in 3 ml ice cold TCA (7%). The resulting precipitate was centrifuged, the pellett washed twice with 7% TCA, then resuspended in a 3 : 1 mixture of ethanol–ether and finally in ether. The residue obtained after ether extraction was hydrolyzed with $HClO_4$ (1.5 mol/l) in boiling water for 30 min. Following filtration and addition of 5 ml scintillation fluid, the radioactivity in 1.5 ml portions of the filtrates was determined as described previously. DNA determination was simultaneously performed from aliquots of this same filtrate. Results were expressed as dpm/μg DNA.

3. *In vitro test system.* Fractions disclosing *in vivo* biological activity were tested under *in vitro* conditions as well. *In vitro* test systems included thymocyte (5×10^6 cells/ml) and bone marrow cells (3×10^6 cells/ml) cultured at 37 °C in a Parker TC 199 medium as well as short term lung organ cultures. In the latter case, SIMNETT's original method [12] was used and the test performed on 48 h lung explants. *In vitro* biological activity of the fractions tested was assessed by measuring the rate of ^{3}H-TdR incorporation into DNA of cells of the different test systems.

Autoradiography

In order to get some insight into the distribution of the *in vivo* administered label among cell populations of the lung tissue, autoradiography was occasionally performed on a few pulmonary tissue samples. The technique included the use of an Ilford K6 emulsion, an exposure time of 3–5 weeks, and a methylgreen-pyronin staining procedure.

Results

The DEAE Sephadex A-50 chromatographic pattern of the ultrafiltered crude extract proved to be highly reproducible. By monitoring optical activity of the eluate at 280 nm, the extract could be resolved into 9, more or less separated elution peaks (Fig. 1). Substances represented by these peaks were collected in four pooled fractions indicated as D_1, D_2, D_3 and D_4, in the figure. Biological activity of the individual fractions was tested under *in vivo* conditions as described in Methods. Presuming that test substances were absorbed *in toto* from the peritoneal cavity and distributed primarily in the extra-

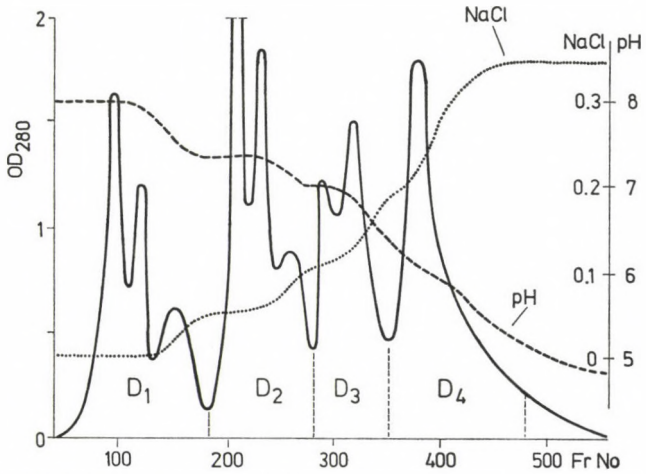

Fig. 1. DEAE Sephadex A-50 chromatogram of a 10 000–100 000 Dalton ultrafiltrate prepared from aqueous extract of bovine pulmonary tissue. Dotted and broken lines indicate salt and pH gradient, respectively. Left-side ordinate: OD_{280}; right-side ordinate: salt concentration (inner side) and pH (outer side), of the elution buffer. Abscissa: number of fractions. D_1–D_4 indicate pooled DEAE Sephadex fractions used in biological test systems

cellular space, the *in vivo* extracellular concentration could be estimated as 50 μg protein/ml after the first dose, and 200 μg protein/ml after the full dose. It should be noted that single doses, even if they were repeated, failed to elicit a biological action.

Table I summarizes results of the *in vivo* screening tests. Substances in the pooled fractions D_1, D_2 and D_4 were found essentially ineffective. On the other hand, intraperitoneal administration of substances in fraction D_3 regularly depressed incorporation of the label, by cca. 35%.

Fraction D_3, the only one that disclosed a significant biological activity in the *in vivo* screening test was further purified on a CM Sephadex C-25 column and yielded 13 elution peaks as demonstrated in Fig. 2. The substances represented by these peaks were pooled in four separate portions indicated

in the figure as fractions C_1, C_2, C_3 and C_4. Their biological activity was tested individually. Provided that the conditions mentioned in connection with fraction D_3 were met, the extracellular concentration of the tested material could be estimated at 30 μg protein/ml after the first dose, and at 120 μg protein/ml after the full one. Results of biological testing are compiled in Table II. The data clearly demonstrate ineffectiveness of the substances in fractions C_2, C_3 and C_4. On the contrary, administration of fraction C_1 which, as it is shown in Fig. 2, also included a component that was evidently excluded from the gel bed (eluted practically at V_0), resulted in a marked inhibition of labeled precursor incorporation into extracted lung DNA. It was also

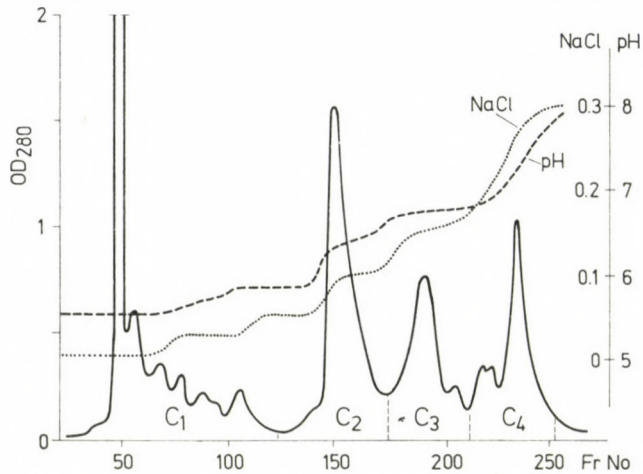

Fig. 2. CM Sephadex G-25 chromatogram of the biologically active D_3 fraction indicated in Fig. 1. Dotted and broken lines as well as coordinates correspond to those indicated in Fig. 1. C_1–C_4 represent pooled CM Sephadex fractions used in biological test systems

Table I

Effect of in vivo administered DEAE Sephadex fractions (50 mg protein/kg body weight) on precursor incorporation into lung tissue as measured by the screening test

Fraction	dpm/mg wet weight	Deviation from the control (%)	p
Control	114 ± 30.9	—	—
D_1	96 ± 41.4	−16	$p > 0.05$
D_2	93 ± 27.1	−18	$p > 0.05$
D_3	75 ± 15.1	−34	$p < 0.05$
D_4	92 ± 23.9	−19	$p > 0.05$

Table II

Effect of in vivo administered CM Sephadex fractions (30 mg protein/kg body weight) on precusor incorporation into DNA of lung and partly of spleen and kidney tissues

Target organ	Fraction	dpm/μg DNA	Deviation from the control (%)	p
Lung	control	116 ± 32.2	—	—
	C_1	58 ± 17.3	−50	$p < 0.05$
	C_2	107 ± 28.2	−08	$p > 0.05$
	C_3	123 ± 36.4	+06	$p > 0.05$
	C_4	93 ± 21.6	−20	$p > 0.05$
Spleen	control	137 ± 44.2	—	—
	C_1	124 ± 42.7	−09	$p > 0.05$
Kidney	control	86 ± 18.8	—	—
	C_1	84 ± 17.6	−03	$p > 0.05$

obvious that fraction C_1, while strongly inhibitory in the lung, remained essentially ineffective in the spleen and kidney, indicating its tissue-specific action (Table III).

Tissue specificity of the action of fraction C_1 applied in two concentrations was demonstrated under *in vitro* conditions as well (Table III). Fraction C_1 inhibited labeled precursor incorporation into DNA of the lung explants

Table III

Effect of two doses (100 and 500 μg protein/ml) of C_1 fraction on labeled precursor incorporation into DNA of different in vitro culture systems

Target culture	Fraction	Dose μg protein/ml	dpm/μg DNA	Deviation from the control (%)	p
Lung	control	—	513 ± 67.1	—	—
	C_1	100	380 ± 41.8	−26	$p < 0.05$
	C_1	500	293 ± 37.1	−43	$p < 0.05$
Thymocyte	control	—	3471 ± 363	—	—
	C_1	100	3799 ± 309	+09	$p > 0.05$
	C_1	500	3518 ± 254	+01	$p > 0.05$
Bone marrow	control	—	4947 ± 580	—	—
	C_1	100	5247 ± 709	+06	$p > 0.05$
	C_1	500	4983 ± 484	+01	$p > 0.05$

in a dose-dependent manner. On the other hand, the same fraction was completely ineffective in both doses, and in both of the remaining cultures.

Autoradiography revealed that incorporation of the labeled thymidine was almost exclusively confined to the nuclei of cells covering the surface of the alveoli (not shown). Consequently, the effect of the active agent(s) responsible for the inhibition of thymidine incorporation might thus be directed only to these cells.

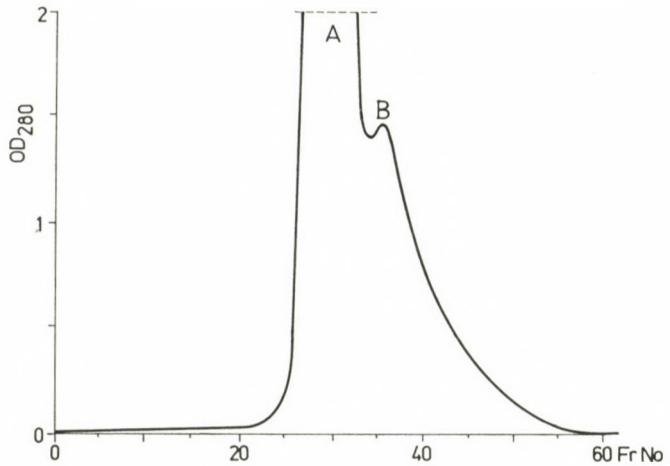

Fig. 3. Sephadex G-75 chromatogram of the biologically active C_1 fraction indicated in Fig. 2. Ordinate: OD_{280}; abscissa: number of fractions

The finding that components included in the biologically active fraction C_1 could hardly be separated on a Sephadex G-75 column might be interpreted by assuming that the molecular weight of its components (Fig. 3) falls, by and large, into an identical range. Substances represented by the poorly separated A and B fractions had an approximate molecular weight of 30 000–50 000 Daltons as measured on a calibrated Sephadex G-75 column. Molecular weight of the components scattered around 40 000 Dalton as determined by ultracentrifugation.

As to the chemistry of the active ingredient(s) in fraction C_1, the following observations argue that it might have been a protein: (a) solutes in the active fraction had a molecular weight of certainly more than 10 000 and less than 100 000 Dalton (ultrafiltration), with 40 000 Dalton as the most likely molecular mass (Sephadex G-75 chromatography and ultracentrifugation); (b) fraction C_1 was found to be largely composed of Folin positive components; (c) the UV absorption spectrum of ingredients in the active fraction was observed to be typical for proteins with a 280 to 260 nm optical activity ratio of 1 : 4; (d) fraction C_1 left standing accidentally at room tem-

perature for 24 h had lost its biological activity; (e) heating of the active fraction at 60 °C for 20 min resulted in precipitation of a significant proportion of its protein content with a concomitant loss of its biological activity.

Discussion

Our present knowledge is uncertain and rather limited in regard of the mechanism of action, and especially the chemistry of the molecules involved in the intricate process of regulation of cell proliferation in pulmonary tissue. The enormous differences known to exist in the rate of proliferation in various expanding and renewing cell populations in mammalian organisms [17] indicate the selective nature of the underlying regulatory process. This is also supported by the known feature of induced DNA synthesis and subsequent cell proliferation, viz. that they are restricted almost exclusively to the cells of a single cell population [18]. This, on the other hand, implies that the respective mediator molecules must possess some special properties enabling them to act in a tissue and/or cell-line specific manner or, at least, to induce or control tissue and/or cell-line specific regulatory processes. The latter may be accomplished by specific interactions operating between the mediator molecule(s) and some special membrane structure, probably receptors of the electively reacting cell populations [19, 20].

It should be stressed that, unlike in experiments hitherto published [12, 13], the *in vivo* effectiveness of an endogenous agent capable of inhibiting DNA synthesis in pulmonary cells in a tissue specific manner has been demonstrated in this paper. Results obtained in our *in vivo* experiments have been confirmed in *in vitro* test systems as well. Nevertheless, the question concerning the reacting cell population within the pulmonary tissue of heterogeneous cellular composition is difficult to answer at present. According to the admittedly preliminary data of our autoradiographic analysis, DNA synthesis of any significant extent could only be observed in cells situated on the surface of the alveoli. Since histological procedures by which different cell types could have been reliably distinguished were not applied in the present experiments, the different types of pneumocytes as well as the macrophages, all situated on the surface of the alveoli, had to be equally considered as target cells. The effect on macrophages could be sorted out by reasoning that, despite the large number of macrophages present in both the splenic and bone marrow tissues, none of them responded to the agent (Tables II and III). Consequently, only type 1 and 2 pneumocytes remained as possible target cells of the active agent. However, according to literary data, rate of DNA synthesis, and consequently cell proliferation in type 1 pneumocytes can hardly be measured and lags considerably behind that of type 2 alveolar cells [21].

Thus it seems very probable that the observed effect was directed primarily to type 2 cells which synthesize DNA at a substantially higher rate. This assumption is supported by our preliminary autoradiographic findings as well. In view of the demonstrated tissue specificity of the *in vivo* effect, together with the *in vitro* ineffectiveness on bone marrow cell and thymocyte cultures of even large doses of the agent, a toxic etiology of the observed action seems to be highly improbable. We are entirely aware of the pitfalls residing in the use of ^{3}H-TdR incorporation into DNA as a proliferation test [22]. However, most of these pitfalls can be disregarded in the light of the *in vivo* effectiveness of the active agent and of its tissue specific action demonstrated under both *in vivo* and *in vitro* conditions.

On the basis of the observations presented in this paper, it appears justified to presume that aqueous lung tissue extracts contain a factor(s) probably of protein character the *in vivo* DNA synthesis inhibiting effect of which is not toxic and lung tissue specific while, with regard to type 2 pneumocytes, it is cell line specific. Several features of the active agent (domain of its molecular weight, its tissue specific action, its protein-like character, etc.) indicate that it might represent a more extensively purified variety of the agent that carried the biological activity in the crude ultrafiltrate used in HOUCK's *in vitro* experiment [13]. Further characterization of the active component by the application of improved separation procedures and newly developed techniques aimed at isolating the active agent in a chemically pure form, a prerequisite of its full chemical characterization, are in progress in our laboratory.

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RESPONSES OF SUBCLAVIAN BARORECEPTORS OF THE DOG TO CHANGES OF STATIC PRESSURE*

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The pressure-dependent changes in afferent activity in the right aortic and vagal nerves were studied in 45 dogs anaesthetized with chloralose. Static perfusion pressures of 0–200 mmHg were applied in steps of 10 mmHg. Single functional fibre preparations were employed. Results were analyzed using a Digital PDP-12 computer. The threshold pressure for steady discharge was 67 ± 3 (SE) mmHg (range, 40–92). The mean saturation pressure was 145 ± 4 mmHg (range, 112–117). The stimulus-response curve relating the pouch pressure to impulse frequency was sigmoid in shape. The sensitivity of these receptors was 0.20–0.7 impulses/s per mmHg. Thus, the subclavian baroreceptors of the dog have characteristics which are not significantly different from those of the aortic arch and carotid sinus baroreceptors. The effects of perfusing subclavian baroreceptors with venous blood was tested on 10 fibres of nine dogs during the application of controlled static pressures to an isolated pouch containing this receptor area. When compared at the same pouch pressures and action potentials, no differences in impulse activities were found during perfusion with either blood.

It is common knowledge that baroreceptors exist at the bifurcation of the right innominate artery in cats, rabbits, and dogs [2]. However, detailed electrophysiological studies involving the baroreceptors in this area have not been undertaken yet.

ANGELL-JAMES [1] carried out electrophysiological studies on the aortic arch baroreceptors, and to a lesser extent, on subclavian baroreceptors of the rabbit, and failed to notice difference in the impulse frequencies recorded from the right and left aortic nerves. The author, therefore, extrapolated his results obtained from the left aortic nerve, which innervated the bifurcation of the innominate artery. The recorded mean threshold pressure was 50.8 (range 0–118) mmHg, with a mean threshold frequency of 35 (range 0.13–59) impulses/s. The mean saturation pressure was 116 (range 63–158) mmHg, with a mean saturation frequency of 77 (range 35–118) impulses/s. However, in related studies, during which perfusion of the subclavian baroreceptor areas was undertaken, UEDA et al. [21] and TANG [19], reported threshold pressures of 20 ± 8 and 60 mmHg, respectively, for the subclavian baroreceptors of rabbits. There have been no such studies in the dog. The purpose of this study was to undertake discrete perfusion of the subclavian baroreceptors with static pressures while recording electrical activity from single strips of the right aortic nerve or right vagus nerve.

* This investigation was undertaken while the author was on study leave at the Department of Cardiovascular Studies, University of Leeds, Leeds, UK

Methods

Forty-five dogs (16–22 kg) were anaesthetized with chloralose (80 mg/kg) given intravenously through the saphenous vein, after intramuscular premedication with morphine sulphate (0.5 mg/kg). Supplementary doses of chloralose were given regularly throughout the experiment to maintain an even level of anaesthesia.

The trachea was cannulated, and artificial respiration was induced, with a mixture of 40% oxygen in air delivered by a modified Starling Ideal pump at a rate of approximately 18 breaths/min. The thorax was opened in the midline, and positive-pressure breathing was induced by adjusting a 3 cm respiratory resistance. The abdomen was opened, and the adrenal glands were identified and ligated. Hydrocortisone (50 mg) was given intravenously. The ansae subclaviae were exposed and crushed, and the vagi were centrally ligated.

Subclavian pouch preparation

A diagram of the perfusion system as well as the isolation of the subclavian baroreceptor area is shown in Fig. 1. A cannula in the right carotid artery recorded pouch pressures. This cannula also drained blood from the pouch into another cannula returning blood to the dog via the external jugular vein. Branches of the right subclavian artery were ligated, and the circuit was completed by ligating the brachiocephalic artery near its junction with the ascending aorta.

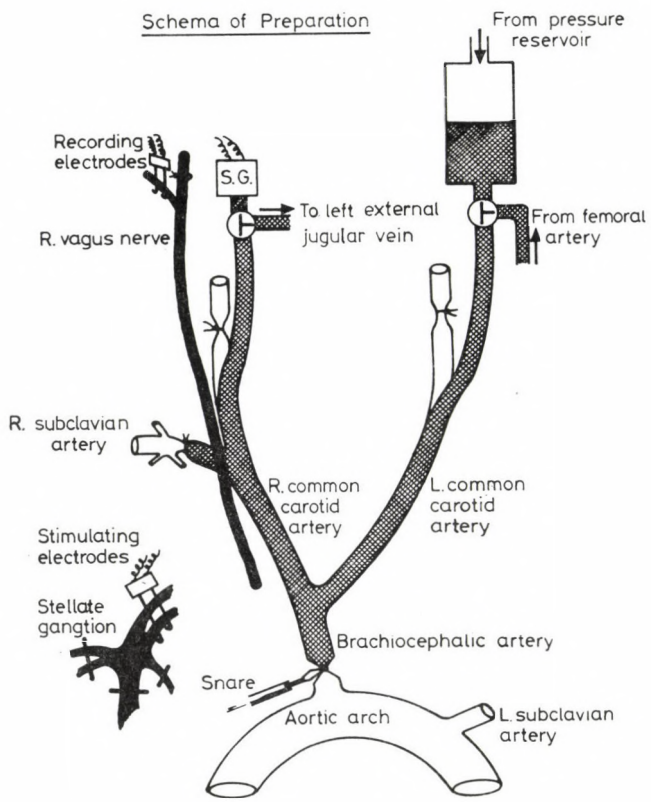

Fig. 1. Scheme of preparation

Haemodynamic measurements

A femoral artery was cannulated; the cannula was connected to a transducer, the pressures from which were displayed on a double-beam oscilloscope. The strain gauge manometers were calibrated in a stepwise manner with saline and mercury manometers, and the zero pressure for each manometer was recorded postmortem as the pressure at the tip of the cannula free in air.

Heart rate was monitored by a cardiotachometer driven by the signal from the amplifier used for recording femoral pressure and the electrocardiogram. The latter was recorded from electrodes applied to the right foreleg, left hindleg, and body.

The pH, pCO_2, and pO_2 were measured using electrode systems (Electronic Industries Ltd., Richmond, Surrey), and the results of blood gas analyses were evaluated according to the scheme of KAPPAGODA et al. [11].

Recording of action potentials

Single strips of the right cervical vagal nerve or the right aortic nerve, bathed in warm (38 °C) mineral oil, were isolated, silver-silver chloride recording electrodes were applied under a dissecting microscope (×10–20). Action potentials were recorded on ultraviolet paper and were also passed through an amplitude discriminator, the output of which was applied to a PDP-12 digital computer for on-line and off-line analysis.

Results

Spontaneous discharge patterns

The pattern of activity recorded from the subclavian baroreceptors of the dog was similar to that already reported for the aortic arch and carotid sinus baroreceptors. These activities were associated with each cardiac cycle and were always pulse-synchronous. The bursts in single fibres had a rate of 4–10 impulses per cardiac cycle and were in phase with the subclavian pressure pulse, the rate and number of action potentials depending upon the configuration of the pressure pulse. The first impulse in each burst occurred early during the rising phase of the pressure wave in the subclavian artery.

As the pressure rose, the number of action potentials increased and reached a maximum when the pressure wave reached the systolic peak, i.e. the interval between action potentials diminished. Thereafter, the interval increased as the blood pressure dropped from its systolic peak, so that there were very few or no impulses at all during the diastolic phase of the pressure wave (Fig. 2). During diastole the impulse discharges ceased at blood pressures that were comparatively higher than the blood pressure levels during systole when impulses were being discharged.

Adaptation time of subclavian baroreceptors

The activity of the receptors during the upstroke of the pressure wave was, of course, heavily dependent upon the rate of rise of the pressure, which was not controlled precisely during the present experiment. Nevertheless it was important to define the time course of adaptation to applied pressures so that measurements of steady-state frequencies could be made.

Subclavian baroreceptor fibres took 30–50 s to adapt to applied static pressures. Adaptation was defined as the time necessary for the initial high frequencies of action potentials, which occurred immediately after the application of pressures, to fall to a lower and steady level that was maintained

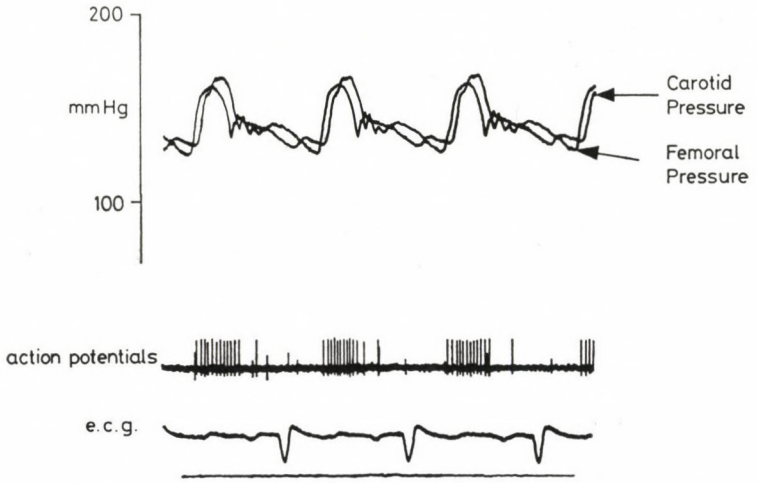

Fig. 2. Spontaneous discharge of subclavian baroreceptors

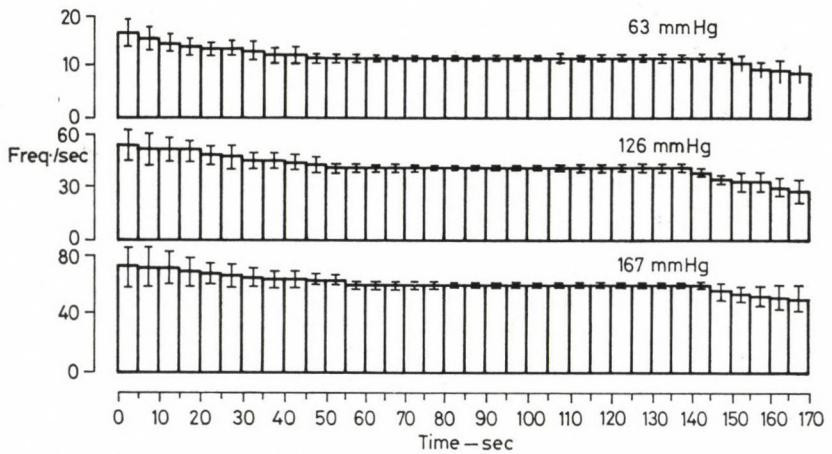

Fig. 3. Adaptation of a typical subclavian baroreceptor perfused at 63, 126 and 167 mmHg

thereafter. The adaptation time varied from one fibre to another nevertheless adaptation was generally accomplished in shorter time periods for lower pressures than for higher pressures; about 30 s were required for pressures around 60 mmHg and 50 s for pressures around 140 mmHg (Fig. 3).

To standardize the results of the present experiments, the steady-state discharges after adaptation were used for evaluation of the results. Sub-

clavian baroreceptors had steady discharges after the first 60 s during the application of any level of pressure, i.e. their adaptation was complete within this time regardless of pouch pressures. Consequently, the mean discharge frequencies were calculated after this time for all levels of pressure.

Determination of the threshold and saturation pressures

The threshold pressures were determined in two ways, and each method was repeated on every fibre examined. First, the pouch pressures were increased in steps of 5 mmHg from zero, and each step was maintained for 2–3 min.

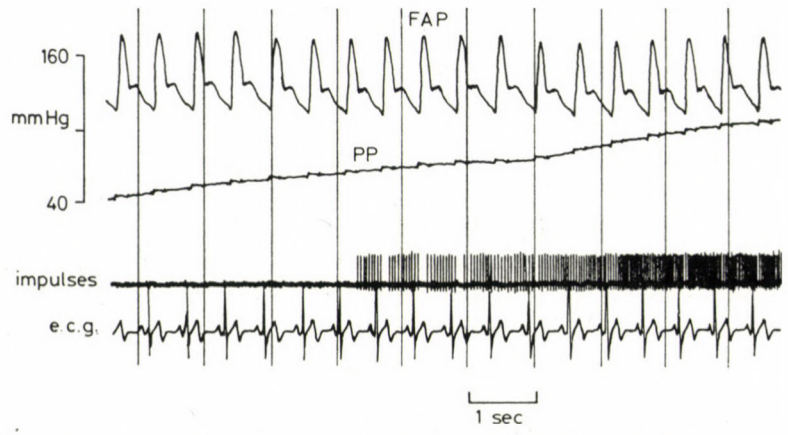

Fig. 4. Determination of threshold pressure by steadily increasing the pouch pressure by approximately 1 mmHg/s

The pressure at which the first maintained impulse occurred was noted. Second, the pouch pressures were increased steadily at rates of approximately 1 mmHg/s and the pressure at which the first maintained impulses occurred was recorded. Figure 4 illustrates a typical experiment using this method. As the pouch pressure rose to 80 mmHg the first maintained impulse occurred.

Values obtained by these two methods were close to one another. The overall calculated mean static threshold pressures were the mean values obtained from the two methods above. Seventy subclavian baroreceptor fibres had a mean threshold pressure of 67 $\pm$ 3 (SE) mmHg (range, 40–92). The mean static threshold discharge frequency was 16 $\pm$ 1.5 (SE) impulses/s (range, 10–30). Figure 5 is a histogram illustrating the distribution of the threshold pressures; the mode is about 68 mmHg.

The pouch pressures were further increased in steps of 10 mmHg until a pouch pressure was reached at which there was no further increase in impulse frequencies. Figure 6 illustrates the results obtained from one experiment in

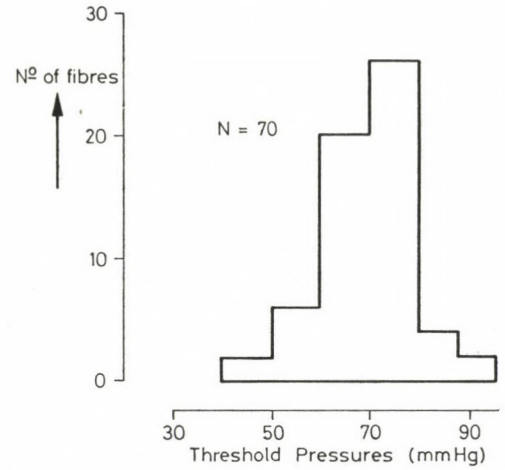

Fig. 5. Mean frequency histogram showing threshold pressure of 70 fibres from the right aortic nerve

which the perfusion pressure was raised from 0 to 150 mmHg. From 0 to 60 mmHg, no activity could be recorded from the fibre. At 70 mmHg, there was a brief burst of activity which adapted rapidly and returned to zero. Above this pressure, the discharges were maintained and increased with increasing pressures until a pressure of 130 mmHg was reached. Beyond this pressure, there was no further increase in the rate of impulses despite increases in pouch pressure. The lowest pressure at which this occurred was regarded as the saturation pressure. The mean saturation pressure was 145 ± 4 mmHg (range, 112–177). The corresponding mean saturation frequency was 42 impulses/s (range, 30–48).

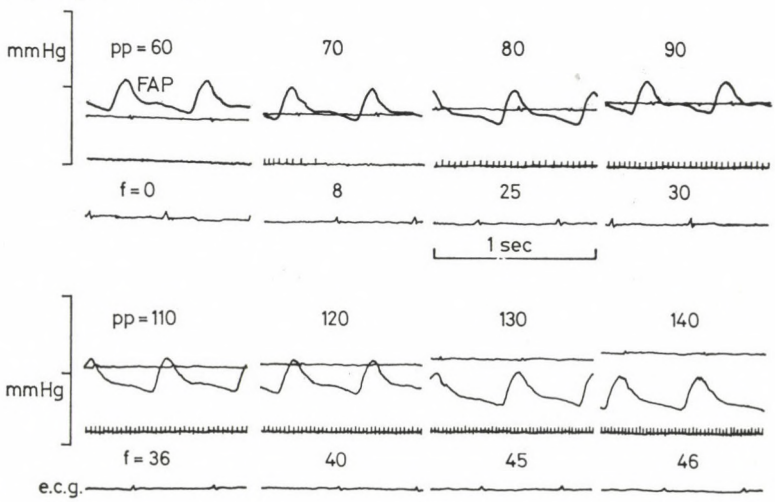

Fig. 6. Effects of altering pouch pressure in steps of 10 mmHg from zero on subclavian baroreceptor impulse activity

Stimulus–response curves were constructed for each subclavian baroreceptor fibre and the curves obtained from four typical fibres are shown in Fig. 7. Decreases in discharge frequencies were never obtained beyond the saturation pressures. Therefore, the shape of the stimulus–response curve beyond the saturation pressure was a plateau. During the application of low static pressures, just above threshold, the frequency increased little for each unit of increased pressure, while further increases induced progressively larger values for the relative changes in frequency and pouch pressure. This

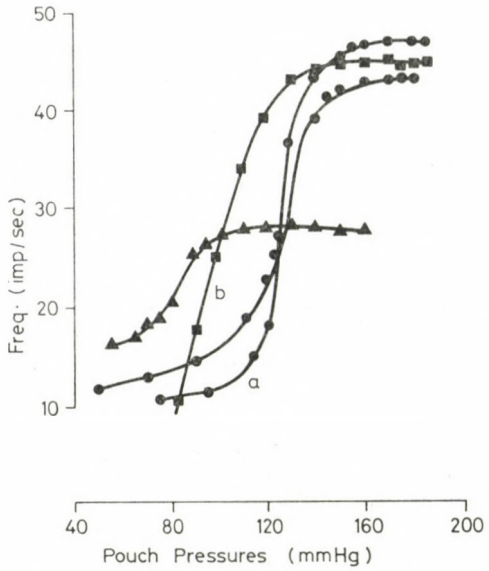

Fig. 7. Graphs showing relationship between impulse activity and pouch pressures for 4 representative subclavian baroreceptor fibres

ratio reached a maximum (maximal sensitivity), and then declined until the saturation pressure was reached. Curves were drawn by the eye and were sigmoid in shape.

The effects of perfusing subclavian baroreceptors with blood of very low oxygen content were tested on 10 fibres of nine dogs. The method used for perfusing the pouch with arterial blood has been described above. The modification was the addition of a second reservoir. One of the reservoirs received oxygenated blood from a femoral artery while the other received venous blood from the femoral vein of the same dog. Both reservoirs were connected to the same air pressure inlet while the drainage from the reservoir was through the same outlet cannula, thereby permitting a switch from one reservoir to another at the same pouch pressure. Any minor variations in the pressures on changing from one reservoir to another were corrected for by adjusting the level of the reservoir.

Blood from either reservoir was passed through the pouch, and recordings of impulse activity using single functional units were made only when the outflow cannula was filled with arterial or venous blood. The pH, pCO_2, and pO_2 of the blood from the outflow cannula in the right common carotid artery

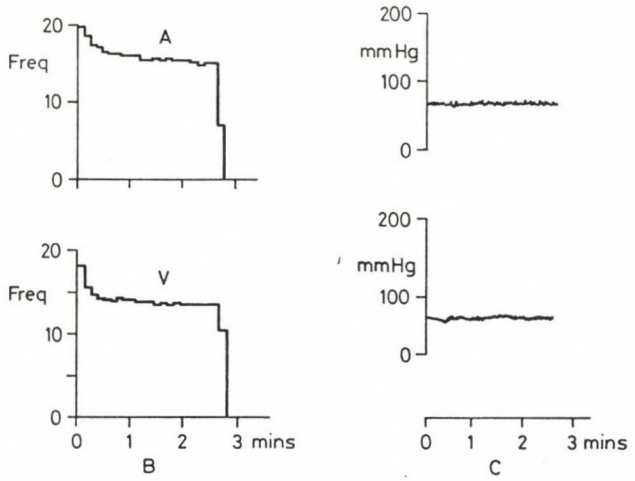

Fig. 8. **Histograms illustrating mean discharge frequencies from a typical subclavian baroreceptor perfused with both arterial and venous blood at a mean pressure of 62 mmHg**

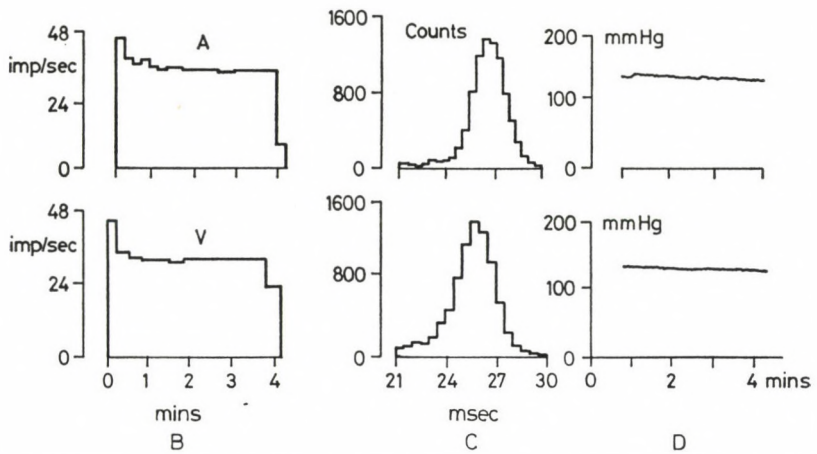

Fig. 9. **Histograms showing discharge frequencies during perfusion with arterial and venous blood at a mean pressure of 125 mmHg**

were continuously determined before, during, and after changes from one reservoir to another. In a few experiments, the oxygen content of the venous blood was further reduced by equilibrating it with a mixture of 40% nitrogen in air. As in the previous experiments, comparisons were based on steady-state discharges of single subclavian baroreceptor fibres only. The range of

Table I

Effects of perfusions with arterial and venous blood

Expt No.	Pouch pressure mmHg	Blood	Frequencies impulses/s	pO_2 mmHg	pCO_2 mmHg	pH
1	60 ± 1	Arterial	11 ± 2	180	35	7.30
		Venous	11 ± 2	34	42	7.22
	90 ± 1.5	Arterial	15 ± 1	177	33	7.31
		Venous	15 ± 2	37	44	7.28
	130 ± 2	Arterial	33 ± 3	185	36	7.32
		Venous	33 ± 2	42	45	7.21
	140 ± 2	Arterial	38 ± 2	181	38	7.33
		Venous	38 ± 2	45	49	7.27
2	62 ± 1	Arterial	14 ± 1.5	185	33	7.32
		Venous	14 ± 2	36	45	7.27
	80 ± 1.5	Arterial	19 ± 2	179	34.6	7.32
		Venous	18 ± 2	46.2	44	7.34
	125 ± 2	Arterial	24 ± 3	180	34.4	7.33
		Venous	24 ± 2	44.8	50	7.27
	145 ± 2	Arterial	36 ± 2	182	34.2	7.32
		Venous	35 ± 2	35.5	47	7.23
3	75 ± 1	Arterial	12 ± 1	185	36	7.32
		Venous	12 ± 2	44	42	7.24
	96 ± 1.5	Arterial	26 ± 2	192	35	7.31
		Venous	26 ± 2	43	45	7.23
	120 ± 2	Arterial	36 ± 2	188	34	7.31
		Venous	36 ± 2	42	45	7.27
	150 ± 2	Arterial	43 ± 3	186	35	7.31
		Venous	43 ± 2	44	47	7.26

Values for pouch pressure and frequencies are mean ± SE

pouch pressures was above threshold pressure but below saturation pressure. The pO_2 level of the venous blood varied from 30 to 50 mmHg over the pressure range.

Figure 8 illustrates a typical mean frequency histogram for a fibre perfused at a pouch pressure of 62 mmHg. The pH of the arterial blood was 7.32 and of the venous blood was 7.27. The pO_2 of the arterial blood was 185 mmHg and of the venous blood was 36 mmHg. The corresponding pCO_2 values were 33 and 45 mmHg. It should be noted that despite the difference

of 149 mmHg in the pO_2 values, the discharge frequencies were the same with both arterial and venous blood. In Fig. 9, the pouch pressure was raised to 125 mmHg. The pCO_2 values were 34.9 and 50 mmHg for arterial and venous blood, respectively, while their respective pH values were 7.33 and 7.27. Here again the discharge frequencies were the same for the two blood perfusions. Table I illustrates the frequencies of discharge of the baroreceptor fibres during perfusion with arterial and venous blood. There were no significant differencies in the frequencies of action potentials.

Discussion

In response to alteration in static pressures, subclavian baroreceptor discharges occurred when the threshold of the individual baroreceptor fibres was reached. The mean threshold pressure for all the fibres examined in the present study was 67 $\pm$ 3 mmHg (range, 40–92). The comparable figures from previous studies on the carotid sinus was 50 mmHg (range, 40–120) [4, 5, 10, 14]. For the aortic arch baroreceptors, mean threshold pressures of 95 mmHg have been reported for the dog [16], and 50.8 mmHg has been reported for the rabbit [1]. The values in the present experiment were not considerably different from those reported by previous investigators for the aortic arch and carotid sinus. The minor differences between the present results and previous results on the carotid sinus and aortic arch baroreceptors may lie in the different preparations used. In the present series, the possible influences on the baroreceptors, either through efferent automatic nerves or humoral substances [7, 14, 15], were eliminated. These procedures have hitherto been ignored.

The mean threshold discharge frequency recorded in the present study was 16 $\pm$ 1 impulses/s (range, 20–40) [1, 4, 5, 8]. Here, again, the differences between the present results and previous studies were not significant, and these differences may also be explained on the basis of the different preparations.

The mean saturation pressure in the present study was 145 $\pm$ 3 mmHg (range, 112–177). Saturation pressures ranging from 100 to 120 mmHg have been reported for the carotid sinus and aortic arch baroreceptor areas [1, 10, 14]. The mean discharge frequency at saturation in the present study was 45 impulses/s (range, 38–48). The comparable value obtained by previous investigators was 55 impulses/s (range 40–70) [1, 8]. These overlapping values indicate that there are probably no structural differences between the baroreceptor elements themselves.

The sensitivity of the subclavian baroreceptors was calculated in the linear range (i.e., above threshold and below saturation), and the value ranged from 0.2 to 0.7 impulses/s per mmHg. Static sensitivities have been calculated

for the carotid sinus baroreceptor units and was found to range from 0.29 to 0.50 impulses/s per mmHg [4, 5, 10, 14]. Static sensitivities ranging from 0.4 to 1.8 impulses/s per mmHg have been reported for the aortic arch baroreceptor units [1, 8]. These values overlap, providing further support for the hypothesis that there are probably no basic differences between the receptors per se. The sensitivity recorded from any experiment depends to a large extent on the nature of the preparation and the size of step changes in pressure from one experiment to another.

The change in nerve activity from threshold frequency to saturation frequency in the present series of experiments was 120% for mean pouch pressure changes from 40 to 170 mmHg. Changes of 114% have been reported for pressure changes from 75 to 175 mmHg [12]. Other investigators have reported changes of 250% [18]; 564% [13]; and 683% [20], for multifibre preparations of the carotid sinus. The value obtained in the present study was in agreement with that of KOUSHANPOUR and KELSO [12] who employed single fibres in their recordings, as in the present study. The large precentage changes reported by the other authors could be due to recruitment and summation of nervous activities in their multifibre preparations.

A curve relating pouch pressure and subclavian baroreceptor impulse activity showed a linear relation over the pressure range of 50–150 mmHg, with the greatest slope around 110 mmHg. The slope of the curve declined rapidly to zero beyond the saturation pressure. The extent to which a vessel stretches depends to a large extent on the geometry of the vessel wall (i.e. its internal radius and wall thickness), and on the stiffness and tissue composition of the vessel wall. These factors were not evaluated in the present study. However, pressure-deformation and deformation-nerve activity relations have been determined for the carotid sinus and aortic arch baroreceptor areas [6, 12, 17]. These investigators noted that nerve activity varied linearly with deformation between pressures of 75 and 175 mmHg, with the slope of the curve rapidly attaining zero above 200 mmHg. The shape of the curve relating subclavian pouch pressures and mean nervous activity in the present study resembles the pressure-volume curves of HALLOCK and BENSON [6]. The saturation pressure occurred at points where the volumes or circumference ceased to increase linearly with applied pressure. If the receptors are assumed to respond predominantly to circumferential strain of the wall [12] there will be less and less nerve activity as the wall strain becomes smaller and smaller near the saturation pressure. Beyond the saturation point no further increase in the wall strain and nerve activity occurs. Alternatively, the saturation may simply be the property of the receptors per se. It is also possible that saturation is a consequence of both mechanisms.

The conclusion that can be drawn from the results involving venous perfusions is that subclavian baroreceptors (and probably all other baro-

receptors) are insensitive to the oxygen tension of the blood perfusing them. This could also further support the assumption that baroreceptors monitor only the changes in pressure and not the changes that occur in the chemical composition of blood, thereby allowing them the remarkable capacity to send accurate information to the cardiovascular centre about the level of pressure within the arteries. This conclusion is consistent with that of BRONK and STELLA [3] who could not detect any differences in impulse activity while perfusing the isolated carotid sinus with either arterial or deoxygenated blood. The apparatus for reporting the chemical composition of blood is probably different from baroreceptors; this function probably resides in chemoreceptors [4]. Furthermore, the results have also shown that any other factors present in the venous blood do not influence the response of the baroreceptors to the application of static pressures. Therefore, the reduction in activity of baroreceptors during sympathetic nerve stimulation cannot be attributed to the anoxia of the receptor terminals [9]. It might also be added that even in disease and at very high altitudes, baroreceptors can be expected to function normally.

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THE MECHANISM UNDERLYING THE DANTROLENE-INDUCED POSITIVE INOTROPIC EFFECT IN GUINEA PIG ATRIAL MYOCARDIUM

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The mechanism of the positive inotropic effect of dantrolene, a skeletal muscle relaxant, was analysed by means of intracellular microelectrode technique and isotonic force transducer in isolated electrically driven left atrial preparations of guinea pigs. Dantrolene had a time-dependent dual effect. First it increased the amplitude of contractions and prolonged the action potential duration. This effect was highly dependent on the stimulation frequency. Mn^{2+} was capable of not only preventing but also abolishing positive inotropic action. After Mn-induced shortening of the action potential duration dantrolene markedly increased it, however the contractile activity was hardly restored suggesting that the drug decreased the repolarizing outward K^+ current. In the K-depolarized myocardium dantrolene induced slow action potentials accompanied by contractions. In the later phase of action the drug successively decreased the amplitude of contractions and shortened the action potential duration, and induced contracture. The two latter phenomena were not observed in Mn^{2+}-treated atria.

The results suggest that dantrolene activates the slow inward Ca^{2+} current by decreasing the outward K^+ current. The late negative effect of the drug could be resulted from a secondary inhibition of the slow inward Ca^{2+} current by increasing the intracellular free Ca^{2+} concentration.

Dantrolene, a direct acting skeletal muscle relaxant, was synthesized by SNYDER and coworkers [43]. The drug has been successfully applied in the treatment of spasticity [6]. Dantrolene inhibits the excitation–contraction coupling of skeletal muscle without changing either neuromuscular transmission [11, 12, 29, 47] or the electrical properties of surface and tubular membrane [9, 17, 41]. This unique property of dantrolene may be due to at least two mechanisms by which the drug exerts its inhibitory action: 1. inhibition of calcium release from the sarcoplasmic reticulum; 2. modification of the charge movement at the triadic junction [3, 11, 16, 35, 36, 44]. BROCKLEHURST's results [3] indicate that dantrolene has no effect on the sensitivity to calcium of the myofibrils and on the calcium uptake of the sarcoplasmic reticulum. It has been shown by DESMEDT and HAINAUT [7] that in fibres micro-injected with ^{45}Ca the calcium efflux was reversibly decreased by dantrolene.

Several observations indicate that dantrolene has only a negligible effect on both cardiac and smooth muscle [4, 10, 12, 14, 36]. CAMERINO and

coworkers [5] demonstrated that dantrolene caused a dose-dependent increase in the tone of the urinary bladder detrusor muscle, which could be antagonized by verapamil. On the other hand, it has been shown that dantrolene decreases the contractile force of isolated rat and guinea pig heart, while the heart rate remains unchanged [2, 34]. In earlier papers [32, 33] we have demonstrated a marked positive inotropic effect induced by high concentrations of dantrolene in guinea pig atrial myocardium. Similar result was obtained by others [17].

The aim of the present study was to analyse the mechanism of the positive inotropic effect induced by 3×10^{-4} M dantrolene in the atrial myocardium of the guinea pig.

Methods

The experiments were carried out on isolated, electrically driven atrial preparations of the guinea pig.

Transmembrane potential recording

The resting and action potentials of the atrial muscle preparations were recorded by means of conventional 3 M KCl-filled glass microelectrodes of 5–15 MΩ resistance. The microelectrode was coupled to the input of a MIKI-1623 cathode-follower amplifier and the recorded potential was displayed and photographed on an EMG-1555 oscilloscope [25, 33]. The maximum rate of rise ($\dot{V}_{max}$) of action potentials was obtained by an electronic differentiator [24, 33]. The preparations were paced by 2 Hz rectangular pulses of 1 ms, double threshold voltage, through an isolating transformer and bipolar platinum electrode. In some experiments the stimulation frequency was varied between 0.3 Hz and 5.0 Hz to determine the dependence of the effect of dantrolene on frequency. In other experiments, dantrolene was tested for its effects on the fast Na^+ channel in atria in which the slow Ca^{2+} channel was blocked by 5 mM Mn^{2+}.

In another set of experiments it was investigated whether dantrolene (3×10^{-4} M) had the ability to induce a slow response in atria in which the fats Na^+ channel had been voltage inactivated by using elevated K^+ (26 mM) Krebs solution (isosmolar substitution of K^+ for Na^+) [23, 30, 33, 39, 40, 42]. Under such conditions the myocardium became inexcitable despite intense electrical stimulation. The K^+-depolarized preparations were paced at a rate of 0.3 Hz. The stimulation voltage was increased up to 10 times the normal threshold.

Contractile force recording

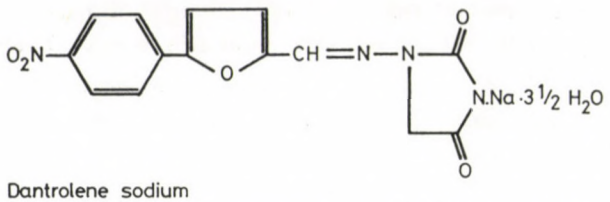
The twitch contractions of the atrial myocardium were measured by an isometric force transducer. The contractions were monitored and photographed on an oscilloscope or recorded on an ink-writing recorder. The resting tension was determined at which the mechanical responses reached the maximum value. The stimulation parameters were the same as described above.

Solutions

The preparations were allowed to equilibrate in Krebs solution for 30 min. The composition of the normal Krebs solution (in mM) was as follows: NaCl, 118; KCl, 4.7; CaCl, 2.5; NaH_2PO_4, 1.0; $MgCl_2$, 1.2; $NaHCO_3$, 24.9; glucose, 11.5, aerated continuously with a gas mixture of 95% O_2 and 5% CO_2; pH was 7.4 and temperature was maintained at 37 °C.

Dantrolene (Norwich-Eaton, New York) was dissolved in 20 per cent propylene glycol to prepare a stock solution of 10^{-2} M. The substance remained dissolved only in warm solution. The final concentration of the drug was achieved by quickly diluting the warm stock solution. Details of the procedure have been described previously [33]. The chemical structure of the drug is shown in Fig. 1.

Drugs used: $MnCl_2$ (Reanal, Budapest, Hungary) and isoproterenol (Isolevin®, Chilag-Chemie AG, Schaffhausen, Switzerland).



Dantrolene sodium

(p-nitrophenyl-furfurylidene-amino-hydantoin sodium hydrate)

Fig .1. Chemical structure of dantrolene

All values presented are means ± S.E.M. The statistical significance of differences from the control was estimated using Student's *t*-test. A p value of less than 0.05 was considered significant.

Results

Effect of dantrolene on the normal action potential and contractility

The effects of dantrolene (3×10^{-4} M) on the electrical and mechanical properties of the guinea pig atria were investigated in relation to time. The results of these experiments are illustrated in Fig. 2. At this concentration the drug exerted a time-dependent dual effect on either twitch contraction amplitude or action potential parameters. In the first phase of action, the drug caused a strong positive inotropic effect. The amplitude of twitches was increased up to three times the control value within 5–15 min (Fig. 2, lower panel). This

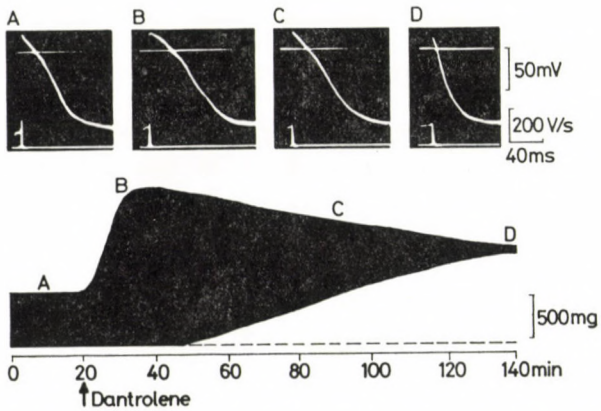


Fig. 2. Time course of 3×10^{-4} M dantrolene-induced changes in contractile force and transmembrane action potential characteristics. Upper panels: intracellular microelectrode recordings. Traces from top to bottom: zero potential level; action potential; maximum rate of rise ($\dot{V}_{max}$). Lower panel: contractions of left atrial preparation recorded at a slow sweep speed. The letters indicate the time of registration of action potentials demonstrated in the upper panels. A: control; B, C, D: effect of dantrolene in relation to time

increment of twitches was accompanied by the lengthening of the action potential duration (by about 30%, at 50% repolarization). Dantrolene slightly decreased the $\dot{V}_{max}$ of action potential without affecting the resting membrane potential (Fig. 2, upper panels). After dantrolene-induced increase of twitches had reached a maximum, they started to decrease gradually and a slow contracture also developed (second phase of action). This negative inotropic effect was associated with a shortening of action potential duration (Table I).

The solvent propylene glycol had no effect in itself on the contractility and electrical properties of the atrial myocardium.

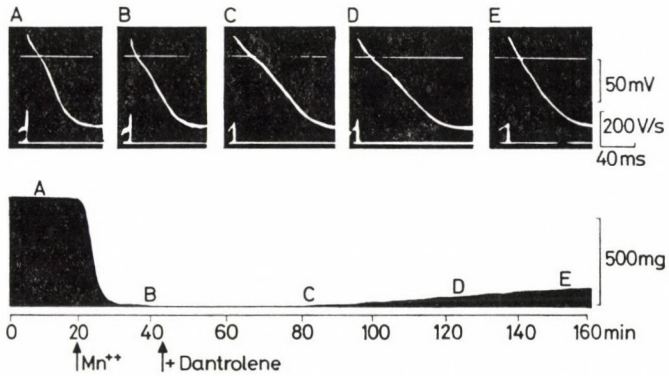


Fig. 3. Influence of 5 mM Mn^{2+} on the time course of 3×10^{-4} M dantrolene-induced change in contractile force and action potential characteristics. Upper panels: intracellular micro electrode recordings. Lower panel: contractions of atrial preparation. A: control; B: effect of 5 mM Mn^{2+}; C, D, E: effect of 3×10^{-4} M dantrolene in relation to time. Mn was presenting throughout the experiment

Influence of Mn^{2+} on the action of dantrolene

In the first series of these experiments, the effect of 3×10^{-4} M dantrolene was studied in atrial myocardium in which the slow Ca^{2+} channel had previously been blocked by 5 mM Mn^{2+}. Mn^{2+} caused electromechanical uncoupling; the mechanical activity was abolished completely and only electrical responses could be recorded (Fig. 3). Under such circumstances, the earlier repolarization (at 20%) of the action potential was accelerated while the late repolarization (at 90%) was prolonged and no change was observed in $\dot{V}_{max}$ or resting potential. After addition of 3×10^{-4} M dantrolene, the action potential duration (at 50%) was prolonged to 165% of the Mn-control value, while contractility was restored hardly from the Mn-blockade. Dantrolene failed to produce contracture and shortening of the action potential duration in Mn-treated atria, i.e. in the absence of freely available slow Ca^{2+} channel (Fig. 3 and Table II).

Table I

Effect of 3×10^{-4} M dantrolene on the transmembrane action potentials in isolated left atrial preparations of the guinea pig. Mean ± S.E.M. of 5 experiments

Treatment	RP (mV)	OS (mV)	$\dot{V}_{max}$ (V/s)	Action potential duration at repolarization (ms)		
				20%	50%	90%
Control	−81.3 ± 0.7	+21.4 ± 1.3	165.2 ± 2.4	19.3 ± 0.7	38.6 ± 0.9	66.1 ± 0.8
Dantrolene 5–15 min	−81.5 ± 1.6	+21.8 ± 1.7	142.2 ± 2.7*	27.3 ± 0.6*	52.4 ± 0.7*	78.0 ± 0.5*
25—50 min	−80.7 ± 1.2	+20.7 ± 1.8	131.0 ± 1.3*	20.1 ± 1.6	39.2 ± 1.5	67.5 ± 1.7
80—120 min	−73.8 ± 2.1*	+12.5 ± 1.7*	124.8 ± 1.5*	5.9 ± 1.2*	14.6 ± 1.4*	34.9 ± 1.3*

* Statistically significant difference from control ($p < 0.05$)
RP = resting potential, OS = overshoot, $\dot{V}_{max}$ = maximum rate of rise of the action potential

Table II

Effect of 3×10^{-4} M dantrolene on the transmembrane action potentials of guinea pig left atria pretreated with 5 mM $MnCl_2$. Mean ± S.E.M. of 5 experiments

Treatment	RP (mV)	OS (mV)	$\dot{V}_{max}$ (V/s)	Action potential duration at repolarization (ms)		
				20%	50%	90%
Control	−79.1 ± 1.3	+23.2 ± 1.4	182.4 ± 0.6	18.2 ± 0.5	32.5 ± 0.8	52.1 ± 0.5
$MnCl_2$	−80.3 ± 0.8	+20.7 ± 0.1*	181.7 ± 0.9	6.3 ± 0.7*	28.3 ± 0.4*	58.3 ± 0.7*
+Dantrolene 15–25 min	−80.5 ± 0.9	+20.5 ± 0.7*	155.2 ± 0.8*	17.2 ± 1.4	41.7 ± 1.2*	75.0 ± 0.3*
35–50 min	−80.7 ± 1.5	+20.9 ± 0.3*	143.0 ± 1.2*	18.0 ± 1.5	50.6 ± 0.9*	83.2 ± 1.1*
90—120 min	−78.2 ± 1.9	+20.8 ± 0.7*	136.1 ± 1.5*	12.8 ± 1.7*	47.3 ± 1.7*	87.6 ± 1.9*
140–160 min	−75.3 ± 1.4*	+19.7 ± 0.4*	127.9 ± 0.7*	10.5 ± 1.6*	32.3 ± 0.5	71.3 ± 1.4*

* Statistically significant difference from control ($p < 0.05$)
RP = resting potential, OS = overshoot, $\dot{V}_{max}$ = maximum rate of rise of the action potential

In the second series of experiments, 5 mM Mn^{2+} was able to antagonise completely the positive inotropic effect caused by dantrolene (Fig. 4).

Induction of slow action potentials by dantrolene

It is generally accepted that drugs which inhibit the repolarizing outward K^+ current, e.g. Ba^{2+} and TEA (tetraethylammonium), can produce slow action potentials in K^+-depolarized myocardial fibres [8, 39, 40]. We

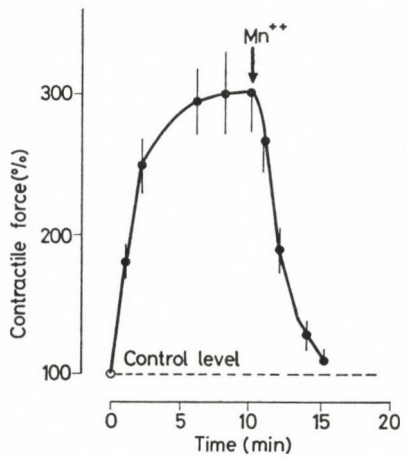


Fig. 4. Effect of 3 mM Mn^{2+} on the 3×10^{-4} M dantrolene-induced positive inotropic effec in left atrial preparations ($n = 5$) of the guinea pig. Values are given as means $\pm$ S.E.M (vertical bars). Lines through the points were drawn by the eye. Dantrolene was added a 0 time

studied whether dantrolene (3×10^{-4} M) had the ability to induce slow action potentials in atria in which the fast Na^+ channel was voltage-inactivated with K^+-rich (26 mM) Krebs solution. Under such conditions the atria became inexcitable despite intense electrical stimulation, i.e. both electrical and mechanical responses were abolished, and only resting potentials (about -45 mV) could be recorded (Fig. 5, B, B'). In 9 of the 12 preparations, the addition of dantrolene (3×10^{-4} M) resulted in slow response action potentials accompanied by contraction within 5–15 min (Fig. 5, C, C'). These slowly rising action potentials had a mean $\dot{V}_{max}$ of 11.5 $\pm$ 1.6 V/s, overshoot of 9.7 $\pm$ 1.3 mV, and duration (at 50% repolarization) of 36.6 $\pm$ 4.1 ms. The amplitude of contractions was nearly equal to that of contractions recorded in normal Krebs solution. Addition of 3 mM Mn^{2+} abolished rapidly (within 2–3 min) the dantrolene-induced slow action potentials and contractions (Fig. 5, D, D').

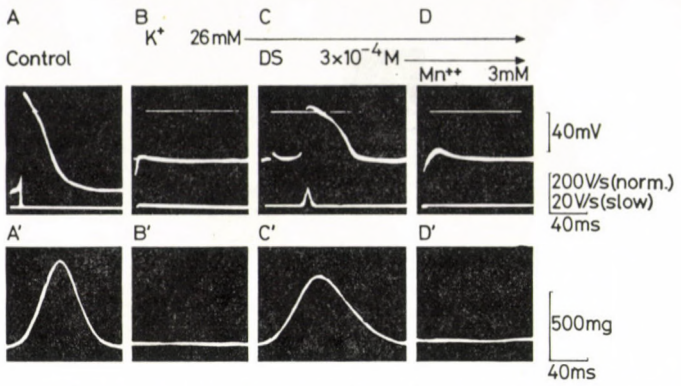


Fig. 5. Induction of slow response action potential and contraction by dantrolene in isolated guinea pig left atrial preparation whose fast Na^+ channel had been voltage inactivated by elevated K^+. A,A': normal action potential and twitch contraction in 4.7 mM K^+ Krebs solution. B,B': application of 26 mM K^+ Krebs solution depolarized the cells to about −45 mV and the preparation became inexcitable. C,C': 3×10^{-4} M dantrolene-induced slow response action potential and contraction. D,D': 3 mM Mn^{2+} completely abolished the slow responses

Effect of dantrolene in relation to frequency

In order to obtain further information on the mechanism of dantrolene action, we examined the increase in dantrolene-induced twitch contractions at different stimulation frequencies (from 1 Hz to 5 Hz), as well as the dependence of dantrolene-induced slow response action potentials and contractions on stimulation frequency. Figure 5 shows force-frequency relationships for the positive inotropic effect of 3×10^{-4} M dantrolene. At each frequency, the action of dantrolene was determined in 5 experiments. The increase in the amplitude of contraction was the most pronounced at low stimulation frequencies ($320.5 \pm 26.0\%$ of control, at 1 Hz). It decreased with increasing frequency of stimulation, and finally, the positive inotropic effect could not be observed at 5 Hz. The increase of frequency also enhanced the contractile force by itself (Fig. 6, inserts), therefore the amplitude of steady state contractions under control conditions were considered as 100% at each frequency.

In three experiments, the dependence on stimulation rate of the dantrolene-induced slow responses was investigated. The control slow responses were recorded at a stimulation rate of 0.3 Hz (Fig. 7, A, A'), and then the frequency was increased gradually. When the increasing frequency of stimulation reached a value of 2.4 Hz, the slow responses disappeared (Fig. 7, D, D').

Comparison of dantrolene with isoproterenol and Mn^{2+}

Since dantrolene exerted a time-dependent dual effect on the electrical and mechanical properties of the guinea pig atrial myocardium, its earlier positive inotropic effect was compared with that of isoproterenol, and the

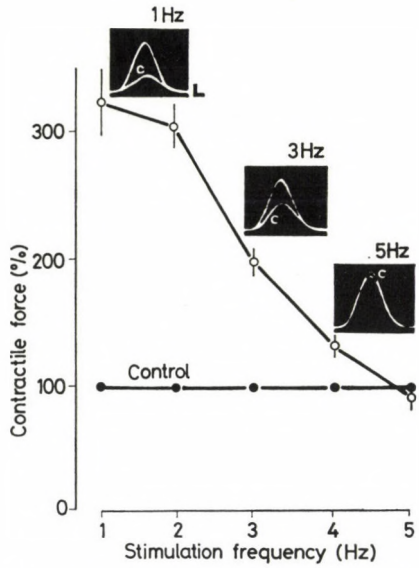


Fig. 6. Effect of 3×10^{-4} M dantrolene on the contractile force of guinea pig left atria in relation to frequency. The amplitude of steady state contractions under control conditions was considered as 100% at each frequency of stimulation. Values are means of 7 experiments ± S.E.M. The dantrolene-induced changes in the size of twitches are shown on the inserts. C: control. Calibrations: Vertical: 250 mg, Horizontal: 20 ms

late negative effect of the drug compared with the Mn-induced negative inotropic effect. As expected, isoproterenol (5×10^{-7} M) induced a strong positive inotropic effect; the contractile force was increased by about 250%, the time to peak tension was shortened by about 18%, the relaxation was accelerated. Isoproterenol increased the $\dot{V}_{max}$ (by about 10%) and enhanced the height of plateau of the action potential. The duration of action potential was prolonged by 42% (at 20% repolarization), while it was shortened by

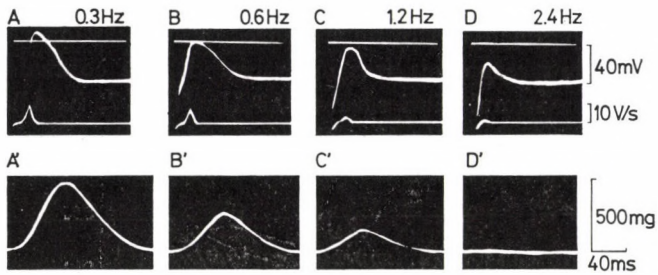


Fig. 7. Dependence of dantrolene induced slow responses on frequency. A,A': control slow response action potential and contraction induced by dantrolene (3×10^{-4} M) in K^+-depolarized atrial myocardium of the guinea pig. B,B' and C,C' and D,D': effect of increasing frequency of stimulation on the slow responses. Records in B through D are from a single impalement

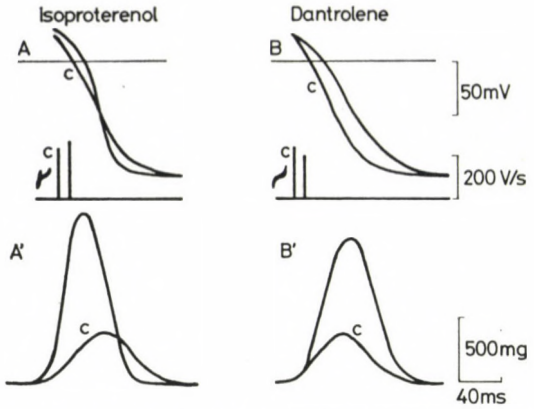


Fig. 8. Comparison of 3×10^{-4} M dantrolene-induced alterations in action potential characteristics and contractile force in the first phase of its action (right panels) with the effect of isoproterenol (5×10^{-7} M), a well known positive inotropic agent (left panels). C: control

16% at 90% repolarization in 5 experiments (Fig. 8, Table III). Under the effect of dantrolene, the contractile force was increased, the time to peak tension increased slightly if at all, while the whole repolarization of the action potential was prolonged and $\dot{V}_{max}$ decreased by about 20% (Fig. 8, right panels).

In the other experiments we compared the effects of dantrolene with that of manganese. Mn^{2+} (5 mM) caused the well-known slow Ca^{2+} channel block accompanied by uncoupling of the excitation–contraction process. The action potentials recorded under these conditions were characterised by a

Table III

Effect of 5×10^{-7} M isoproterenol (IPR) on the transmembrane action potentials of guinea pig left atria. Mean ± S.E.M. of 5 expriments

Treatment	RP (mV)	OS (mV)	$\dot{V}_{max}$ (V/s)
Control	−83.5 ± 1.4	+22.4 ± 1.3	215.8 ± 2.7
IPR	−86.1 ± 1.9	+28.7 ± 1.6*	236.3 ± 2.1*

Treatment	Action potential duration at repolarization (ms)		
	20%	50%	90%
Control	19.2 ± 0.8	34.3 ± 0.5	72.5 ± 0.6
IPR	27.7 ± 0.9*	40.5 ± 0.7*	61.4 ± 0.5*

* Statistically significant difference from control ($p < 0.05$). RP = resting potential OS = overshoot, $\dot{V}_{max}$ = maximum rate of rise of the action potential

shortened plateau and prolonged late repolarization, while no change was observed in the $\dot{V}_{max}$ (Fig. 9, left panels). After a long lasting treatment, dantrolene (3×10^{-4} M) induced contracture. The contractility was diminished or completely abolished, while the whole repolarization of action potential was shortened, the $\dot{V}_{max}$ lowered and the membrane slightly depolarized (by about 4–6 mV) (Fig. 9, right panels).

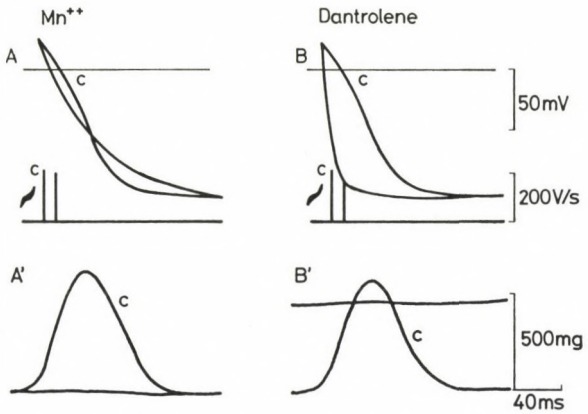


Fig. 9. Comparison of 3×10^{-4} M dantrolene-induced alterations in action potential characteristics and contractile force in the second phase of its action (right panels) with the effect of Mn^{2+} (5 mM), a direct acting slow Ca^{2+} channel blocker (left panels). C: control

Discussion

The results of the present experiments show that dantrolene at a concentration of 3×10^{-4} M has a time-dependent dual effect on the contractile force and electrical properties of the guinea pig atrial myocardium.

The results so far published are inconsistent regarding the effect of dantrolene on cardiac muscle. Several authors reported that dantrolene had only a negligible effect on cardiac muscle [4, 10, 14, 36]. On the other hand, a negative inotropic effect was obsered when high concentrations were used [2, 34]. More recently, similarly to other investigators [17], we demonstrated that high concentrations (10^{-5} M and above) of dantrolene exerted a strong positive inotropic effect on the guinea pig atrial myocardium, which was independent of beta-adrenoreceptors and histamine receptors [32, 33]. The discrepancy between the results obtained in the present study and the observations by others may be explained by the fact that they applied dantrolene at concentrations of less than 10^{-5} M, whereas we used a higher and probably toxic concentration (3×10^{-4} M).

We suppose that the initial transient positive inotropic effect of dantrolene might be due to the stimulation of the transmembrane slow inward Ca^{2+}

current flowing during the plateau phase of normal action potential rather than to any other intracellular event. This concept is supported by the fact that Mn^{2+} was able to completely antagonize this effect. Mn^{2+} is a well known selective blocker of the slow Ca^{2+} channels [15, 26, 37, 45]. Moreover, dantrolene itself induced slow response action potentials and contractions in partially depolarized atrial preparations. It has been shown that drugs inducing slow responses stimulate the transmembrane slow inward current carried mainly by calcium ions [18, 23, 30, 39, 40, 42, 46]. Furthermore, the dantrolene-induced slow responses as well as the positive ionotropic effect were found to be highly sensitive to the frequency of stimulation, i.e. the lower the rate of stimulation, the greater the degree of positive action. This phenomenon could have resulted from a relatively high time constant for recovery of the slow inward calcium system from inactivation [13, 27].

According to PAPPANO's suggestion [39], there are two different mechanisms for the activation of slow Ca^{2+} channels by which drugs (e.g. isoproterenol or Ba^{2+}) may induced slow responses: (i) increase of an ion conductance that carries inward current, (ii) decrease of an ion conductance that carries outward current. In the case of dantrolene induced slow responses, the latter mechanism seems to be validated by the fact that dantrolene markedly prolonged the duration of action potential without restoration of the mechanical activity in atria in which the slow Ca^{2+} channel and contractility had previously been depressed by Mn^{2+}. EHARA and INAZAWA [8] demonstrated that Ba ions intensified the slow Ca^{2+} current by decreasing the membrane shunting conductance occurring in K^+ depolarized myocardium [31]. Figure 8 shows the effect of dantrolene in comparison with that of isoproterenol as a 'direct' (via cyclic AMP) stimulator of the slow inward Ca^{2+} current. Isoproterenol increased the height of plateau and accelerated the late repolarization of the normal action potential (see also in [38]). Recent voltage clamp experiments indicate that an excitation-related sudden increase in $[Ca^{2+}]_i$ enhances the outward K^+ current [1, 19, 20, 22].

The late negative inotropic effect of dantrolene could be conceived as a consequence of its earlier positive inotropic effect, if we suppose that the drug, besides activating slow Ca^{2+} channel, inhibited the Ca^{2+}-efflux from the atrial myocardial cells. DESMEDT and HAINAUT [7] have demonstrated that ^{45}Ca-efflux from barnacle muscle fibres is decreased by dantrolene, whereas ^{45}Ca-influx is unchanged. In this way, dantrolene successively increases the $[Ca^{2+}]_i$ and, consequently, decreases the transmembrane calcium concentration gradient, leading to a lessening of the driving force for calcium ions. A similar explanation was given by KOHLHARDT and coworkers [28] for the biphasic effect of caffeine on transmembrane Ca^{2+} current in cat papillary muscle. This hypothesis is supported by the finding of the short action potential duration and of the contracture. It has also been demonstrated that action

potential duration is controlled by the $[Ca^{2+}]_i$ [1, 19, 20, 21, 22]. As shown in Fig. 9, the dantrolene-induced indirect inhibition of the slow inward Ca^{2+} current flowing during the plateau phase of normal action potential differs from its Mn-induced direct inhibition.

In conclusion, dantrolene depresses the excitation-related outward movement of both potassium and calcium ions. This common effect of dantrolene may account for its earlier positive and late negative inotropic effects.

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РЕЗЮМЕ

ЭЛЕКТРИЧЕСКИЕ СИГНАЛЫ АКТИВНОСТИ СИНУСНОГО УЗЛА У СОБАКИ

Ф. ШОЛТИ, Ш. ЮХАС-НАДЬ и А. ЧАКО

В экспериментах на собаках (22 животных) авторы занимались местной регистрацией активности синусного узла. С помощью маленького униполярного электрода, расположенного вблизи синусного узла, и пользуясь большим электрическим усилением, авторы смогли зарегистрировать раннюю, медленную, маленькую волну, появляющуюся за 20—40 сек перед волной Р. При удалении от синусного узла эта ранняя волна становится все меньше и, наконец, растворяется в локальной волне Р. Местная волна Р. исчезает при временном прекращении деятельности синусного узла, что может быть достигнуто введением аденозина в артерию синусного узла, раздражением блуждающего нерва и инфильтрацией фенолом области синусного узла. Если задавать ритм синусному узлу, раздражая электрическим током предсердие, то ранняя волна тоже не появляется перед волной Р. Зарегистрированная вблизи синусного узла, ранняя волна в самом деле указывает на деполяризацию синусного узла, и является подходящим объектом для изучения роли синусного узла как водителя ритма, а также синоаурикулярной проводимости возбуждений.

РОЛЬ СОДЕРЖАНИЯ МИОКАРДИАЛЬНОЙ ЖИДКОСТИ ДЛЯ ДЕЯТЕЛЬНОСТИ СЕРДЦА

Г. ПОГАЧ, М. КОЛТАИ и ДЬ. ГРОС

В своих предыдущих исследованиях мы показали, что через 2 и 48 час после наступления инфаркта миокарда, вызванного инфузией норадреналина (6 нмоль/кг в мин) в течение 10 мин, а также во время фибрилляции желудочков, продолжительностью 60 мин, при экстракорпоральном кровообращении, развивается значительный интерстициальный отек. С другой стороны, мы вызывали дегидратацию сердечной мышцы, если, после инфузии маннита или панкреатэктомии, вводили глюкозу до тех пор, пока осмотическое давление не достигало 320—470 мОсм/литр. Удалось показать тесную двулинейную корреляцию между диастолическим напряжением левого желудочка и содержанием жидкости в сердечной мышце. Далее, мы показали, что возрастание диастолического напряжения сердечных желудочков влечет за собой уменьшение минутного объема сердца, особенно заметного при нагрузке. Минутный объем сердца бывает существенно уменьшенным при инфаркте миокарда и после панкреатэктомии, вслед за проведением экстракорпорального кровообращения и во время введения маннита.

Клиническое значение имеет наблюдение, согласно которому производительность сердца уменьшается во всех случаях, когда содержание жидкости в тканях миокарда либо не достигает, либо превышает значение 760—790 г в расчете на 1000 г сердечной мышцы.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ СВЯЗИ МЕЖДУ ОСМОТИЧЕСКИМ ГЕМОЛИЗОМ И ТЕМПЕРАТУРОЙ, ВЫПОЛНЕННОЕ С ЭРИТРОЦИТАМИ ВЗРОСЛЫХ ЛЮДЕЙ И НОВОРОЖДЕННЫХ ДЕТЕЙ

Ш. ИМРЕ, Б. ШАРИ, И. ТЁРЁК и Э. ДВОРАЧЕК

Гипотонический гемолиз эритроцитов, как новорожденных детей, так и взрослых людей, более сильно выражен при 4°С, чем при 37°С. В связи с гипотоническим гемолизом эритроциты новорожденных более резистентны по отношению к снижению температуры,

чем эритроциты взрослых людей, подвергнутые такой же обработке. При изображении осмотического гемолиза клеток в зависимости от изменений температуры, имеется возможность определить транзиторную температуру мембраны красных кровяных клеток, благодаря чему можно охарактеризовать микровязкость мембраны. Изображая с зависимости от температуры гемолиз, происходящий при помещении эритроцитов в раствор хлористого натрия с осмотическим давлением 150 мОсм, мы нашли, что показатель преломления, представляющий транзиторную температуру на прямой функции Аррениуса, находится при 25°С, как в случае эритроцитов новорожденных, так и взрослых людей. Тождественность транзиторной температуры указывает на схожесть микровязкости мембраны эритроцитов новорожденных и взрослых людей.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ДЕЙСТВИЯ БЕНЗОЛА, ТОЛУОЛА И *m*-КСИЛОЛА В ЭКСПЕРИМЕНТАХ НА КРЫСАХ

К. А. ПАКШИ, Й. МОЛНАР, М. НАРАИ и ДЬ. БАРЦА

В опытах на крысах авторы изучали седативное действие, а также влияние на мотивационное поведение трех растворителей (бензол, толуол и *m*-ксилол), которые вводились интраперитонеально. Применялись следующие тесты: ***open field*** (эксплоративная деятельность, локомоция), колесо для измерения двигательной активности (активность бега), трубочный тест и тест с наклонной пластинкой (мышечная слабость, нарушения координации).

Уже в малых концентрациях все три вещества вызывали мышечную слабость и атаксию. В этом отношении бензол вызывал самый сильный эффект. Действие же всех трех растворителей на двигательную активность было очень разным. Если бензол и особенно толуол вызывали возбуждение центральной нервной системы, то у *m*-ксилола такого действия не отмечали. Зная из человеческой практики о том, как действуют эти вещества, можно сделать вывод, что система исследований, применяемая у крыс, может оказаться полезной для своевременной сигнализации таких острых вредных влияний, какие ожидаются у человека.

УТЕРО-ИНГИБИН — ВЫДЕЛЕННЫЙ ИЗ ПЛОДНОЙ ЖИДКОСТИ ФАКТОР, ИНГИБИРУЮЩИЙ СОКРАЩЕНИЯ МАТКИ

А. ПАЙОР, Й. ГРОФ, М. ИДЕИ, Я. МЕНЬХАРТ и Б. ЖОЛНАИ

Авторы изучали действие смеси проб околоплодной жидкости, полученных амниоцентезом во втором триместре беременности, в условиях ***in vitro*** на препарате изолированной беременной матки крыс. Установили, что околоплодная жидкость имеет одновременно как стимулирующую, так и тормозящую активность. С помощью хроматографии (на геле Сефадекс G-25, ионообменной СМ-Сефадекс G-25 и бумажной) удалось выделить и очистить компонент плодных вод, ответственный за ингибирование маточных сокращений. Фракция, содержащее действующее вещество (утеро-ингибин), тормозит сокращения миометрия беременной матки человека, препарата кишки крысы, а также вызванные электрическим раздражением сокращения ***in vitro*** препаратов ***vas deferens*** мыши и снижает их базальный тонус.

Можно предположить, что, в обеспечении состояния покоя матки в период беременности, играет роль какой-то, до сих пор не извстный, эндогенный фактор.

СУДЬБА СЕКРЕТОРНЫХ БЕЛКОВ ПАНКРЕАТИЧЕСКОГО ПРОИСХОЖДЕНИЯ В КРОВЯНОМ РУСЛЕ У СОБАК И КРЫС

М. ПАПП, Г. ВАРГА, Г. ФОЛЛИ и В. ТЁРЧВАРИ

Авторы собирали свежий панкреатический сок у животных-доноров (собак и крыс), предварительно стимулируя деятельность поджелудочной железы у них внутривенным введением секретина и панкреозимина (4 Е/кг веса тела). После определения в панкреати-

ческом соке уровня белка и активности липазы и амилазы, его вводили в кровяное русло животного-реципиента того же вида. Определяли пространство распределения, время биологического полураспада и выведение с мочой и желчью секреторных белков, повторное их выделение с панкреатической жидкостью и возвращение из межтканевого пространства через грудной лимфатический проток. Фроме того, определяли объем плазмы у реципиентов-животных.

Пространство распределения как липазы, так и амилазы у крыс совпало с объемом плазмы. У собак биологическое время полураспада амилазы было продолжительнее, чем липазы. Если в моче и желчи у собак количество выделенных энзимов было ничтожно мало, то количество энзимов в моче крыс достигло 10% от введенного количества. У собак, введенные в кровоток, липаза и амилаза повысили уровень липазы и амилазы в панкреатической жидкости, и как у крыс, так и у собак уровень и количество белков в панкреатической жидкости. Часть энзимов, покинувших кровяное русло, возвращается в кровообращение через грудной лимфатический проток.

ВЛИЯНИЕ МАЗИНДОЛА НА ПИЩЕВОЕ ПОВЕДЕНИЕ И НА ФУНКЦИЮ НОРАДРЕНАЛИНА В РАЗЛИЧНЫХ УЧАСТКАХ ГОЛОВНОГО МОЗГА У КРЫС

Л. ХАЛМИ, Ч. НЯКАШ и Ю. ВАЛЬТЕР

На крысах, помещенных в новые условия и не получавших пищу в течение 24 часов, мы изучали влияние интраперитонеально введенного мазиндола на пищевое поведение животных, а также на исчезновение ^{3}H-норадреналина из разных областей головного мозга. В последнем случае мазиндол вводился интрацеребровентрикулярно.

Мазиндол сильнее подавлял пищевое поведение, чем прием пищи. Это означает, что пищевое поведение более чувствительно указывает на понижающее аппетит действие лекарства, чем прием пищи. В дозе 30 мг/кг мазиндол повышал исчезновение ^{3}H-норадреналина из медиального гипоталамуса, но не оказывал действия на обмен норадреналина в других областях мозга.

Результаты настоящих экспериментов указывают на то, что действие мазиндола на обмен ^{3}H-норадреналина является разным в различных участках мозга.

ЧАСТИЧНОЕ ОЧИЩЕНИЕ ИЗ ЛЕГОЧНОЙ ТКАНИ КРУПНОГО РОГАТОГО СКОТА БЕЛКА, СПОСОБНОГО ИНГИБИРОВАТЬ *IN VIVO* СИНТЕЗ ДНК В ЛЕГОЧНЫХ КЛЕТКАХ МЫШИ

З. МАРЧЕК и Я. МЕНЬХАРТ

Настоящее сообщение знакомит с частичным очищением и характеристикой эндогенного фактора, который тканеспецифически ингибирует в условиях *in vivo* синтез ДНК в клетках легочной ткани мышей.

Данное активное вещество очищали из водного экстракта легочной ткани крупного рогатого скота и крыс, применяя комбинацию ультрафильтрации и различной хроматографической техники.

Вещество, ответственное за отмеченный эффект, термолабильно и, вероятно, является белком с молекулярнУм весом около 40 000. На основании определенных до настоящего времени химических и физических свойств, а также биологического действия, выделенное из легких вещество, по-видимому, является эндогенной пролиферационной ингибиторной молекулой, которая оказывает действие на легочные эпителиальные клетки. Эта молекула имеет халоноподобные свойства.

ОТВЕТЫ С БАРОРЕЦЕПТОРОВ ПОДКЛЮЧИЧНОЙ АРТЕРИИ НА ИЗМЕНЕНИЯ СТАТИЧЕСКОГО ДАВЛЕНИЯ У СОБАКИ

А. У. КАДИРИ

В экспериментах, проведенных на 45 наркотизированных собаках, изучали изменения афферентной активности блуждающего нерва и правого аортального нерва в зависимости от давления. Статическое перфузионное давление повышали ступенеобразно на

10 мм рт. ст., в пределах от 0 до 200 мм рт. ст. Применяли функциональный препарат на изолированном нервном волокне. Результаты анализировали с помощью дигитального компъютера PDP-12. Постоянная импульсная активность начиналась при пороговом давлении, равном 67 ± 3 (SE) мм рт. ст. (в области давлений от 40 до 92 мм рт. ст). Среднее сатурационное давление было 145 ± 4 мм рт. ст. (между 112—117 мм рт. ст.) Сравнивая давление в мешке, образованном из подключичной артерии, с частотой импульсов, получили кривую раздражениеответ сигмовидной формы. Чувствительность рецепторов была 0,2—0,7 имп/сек/мм рт. ст.

Таким образом, барорецепторы подключичной артерии у собаки не отличаются существенно от барорецепторов дуги аорты и каротидного синуса.

На 10 нервных волокнах у 9 собак изучали действие перфузируемых венозной кровью барорецепторов подключичной артерии на частоту возникновения импульсов, применяя контролируемое статическое давление и изменяя давление в содержащем рецепторное поле изолированном мешке подключичной артерии. Перфузию артериальной и венозной крови сравнивали с давлением в мешке, и регистрировали эффект на изолированных волокнах правого аортального нерва. Разницы между значениями среднего давления в мешке, при перфузии артериальной или венозной кровью, не наблюдали.

МЕХАНИЗМ, ЛЕЖАЩИЙ В ОСНОВЕ ПОЛОЖИТЕЛЬНОГО ИНОТРОПНОГО ДЕЙСТВИЯ ДАНТРОЛЕНА НА МЫШЦУ ПРЕДСЕРДИЯ СЕРДЦА МОРСКОЙСВИНКИ

Я. МЕЙСАРОШ, В. КЕЧКЕМЕТИ и Й. СЭГИ

Мы изучали механизм положительного инотропного действия мышечного релаксанта дантролена на препарате изолированной мышцупредсердия морской свинки. Исследования производились техникой внутриклеточных микроэлектродов и с помощью измерения изометрических сокращений. Дантролен оказывает время-зависимое двойственное действие. В первой фазе действия препарат увеличивает амплитуду сокращений и продолжительность потенциалов действия. Этот эффект очень чувствителен по отношению к частоте раздражения и к ионам магния. Вызванное дантроленом удлинение продолжительности потенциалов действия наблюдалось и в присутствии ионов магния. В миокарде, деполяризованном ионами калия, дантролен вызывал появление медленных потенциалов действия. В поздней фазе действия дантролена амплитуда сокращений постепенно снижается и развивается медленная контрактура, которая сопровождается сильным укорочением продолжительности потенциалов действия.

На основании полученных результатов мы приходим к выводу, что дантролен активизирует медленное течение ионов кальция и увеличивает внутриклеточную концентрацию свободного калция.

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