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Influence of rat plasma and of various cations on the anti-eledoisin activity of morin

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1. The musculotropic activity of eledoisin on the isolated guinea-pig ileum is antagonized by morin, a flavonoid derivative.

2. The anti-eledoisin activity of morin is abolished by fresh rat plasma and by deproteinized rat plasma, but not by deionized plasma.

3. Cupric acetate can restore the inhibitory property of the deionized plasma and can also inactivate morin.

4. This effect of rat plasma and of cupric acetate is inhibited by (−)-penicillamine and by N-acetyl-(±)-penicillamine.

5. It is concluded that, when in contact with rat plasma, morin is probably inactivated as a consequence of the formation of a complex with the plasma copper.

Since 1960, various compounds have been shown to inhibit some of the actions of bradykinin (Mariani, 1961 ; Collier, 1961 ; Garcia-Leme & Rocha e Silva, 1965) and angiotensin (Schaper, Jageneau, Xhonneux, Van Nueten & Janssen, 1963 ; Godfraind, Kaba & Polster, 1966 ; Gascon & Walaszek, 1966). The first compound which has been found to antagonize the actions of eledoisin is the flavonoid derivative morin (Fig. 1). On the guinea-pig ileum this substance reversibly but non-competitively inhibits the actions of eledoisin in concentrations which do not effect the responses to angiotensin and bradykinin and which slightly potentiate the actions of acetylcholine (Gascon, 1968a). Morin also inhibits the effects of eledoisin on isolated rabbit atria (Gascon, 1968b) but does not significantly reduce the hypotensive action of eledoisin in the rat. In order to elucidate this lack of activity *in vivo*, we studied the influence of rat plasma and of various cations on the anti-eledoisin activity of morin *in vitro*, using the isolated guinea-pig ileum.

Methods

Guinea-pig ileum

Strips 2–3 cm long suspended at 30° C in a 10 ml. bath of Tyrode solution gassed with a mixture of 95% oxygen and 5% carbon dioxide. Eledoisin was added at

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constant intervals of 5 min and left in contact with the smooth muscle for 60 sec. The contractions induced by the agonist were recorded on a smoked drum with a frontal writing lever. Responses were first obtained to a series of four doses of eledoisin, each sequence being repeated two or three times. Morin was added to the perfusion medium in a final concentration of 2 or 4 μg/ml. and kept in continuous contact with the preparation. The influence of rat plasma and of various cations was evaluated at a concentration of 4 ml./l. and 6×10^{-6}M, respectively.

Preparation of rat plasma

Fresh plasma. Fresh plasma was obtained by centrifugation of heparinized blood withdrawn from the abdominal aorta of the ether-anaesthetized animal.

Deproteinized plasma. To obtain deproteinized plasma, perchloric acid 10% was added to the fresh rat plasma until no more precipitation occurred ; the mixture was then kept at 5° C overnight before being centrifuged and neutralized.

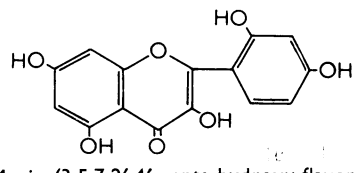
Deionized plasma. Deproteinized plasma was allowed to run through a column of Amberlite MB1, an intimate mixture of Amberlite IR-120 and Amberlite IRA-400.

Blood pressure experiments

Male Sprague–Dawley rats weighing 150–200 g were pretreated with N-acetyl-(±)-penicillamine (4 mg/day per rat, i.p.) for periods ranging from 5 to 15 days. The rats were treated with guanethidine 10 mg/kg (Miele & DeNatale, 1965), 24 hr before the blood pressure recordings. The animals were then anaesthetized with Nembutal (40 mg/kg, i.p.) and the systemic arterial blood pressure was recorded from the carotid artery by means of a Statham pressure transducer (model P23) on a Grass polygraph. The agonists were dissolved in isotonic saline and injected in a constant volume of 0.2 ml. into a cannulated jugular vein. In the first part of the experiments, responses were obtained to eledoisin and acetylcholine. After this control period, morin 40–50 mg/kg was injected intraperitoneally in a 50% alcoholic solution. Five minutes later, the action of each agonist was re-evaluated over a period of 60 min.

Drugs

Eledoisin (ELD-950, Sandoz), acetylcholine bromide (Eastman Organic Chemicals), morin dihydrate (Aldrich), N-acetyl-(±)-penicillamine (Nutritional Biochemical Corporation), (−)-penicillamine (Nutritional Biochemical Corporation), cupric acetate (Merck), cadmium acetate (Merck), cobalt acetate (Merck), zinc



Morin (3,5,7,2′,4′ penta hydroxy flavone)

FIG. 1. Chemical structure of morin.

acetate (Merck), strontium acetate (Merck), nickel acetate (Merck), aluminium acetate (Merck), lead acetate (Merck) and guanethidine sulphate (Ismelin, Ciba). The drug concentrations are expressed in terms of their salts.

Statistical analysis

The statistical analyses used were: determination of the correlation coefficient, regression analysis and the Student *t* test, which was applied to the slope of the regression curves.

Results

Guinea-pig ileum

In the first series of experiments, we studied the influence of fresh rat plasma on the anti-eledoisin activity of morin. The results (Fig. 2) indicate that morin (2 and 4 μg/ml.) produces a marked reduction in the musculotropic activity of eledoisin. Furthermore, it can be observed that when fresh rat plasma is added to the perfusion mixture before the addition of morin (2 and 4 μg/ml.), the latter compound loses its inhibitory effect completely. Similar results were obtained when deproteinized plasma was used (Fig. 3). When deionized plasma was added to the Tyrode solution, however, morin was still able to antagonize the musculotropic activity of eledoisin (Fig. 4).

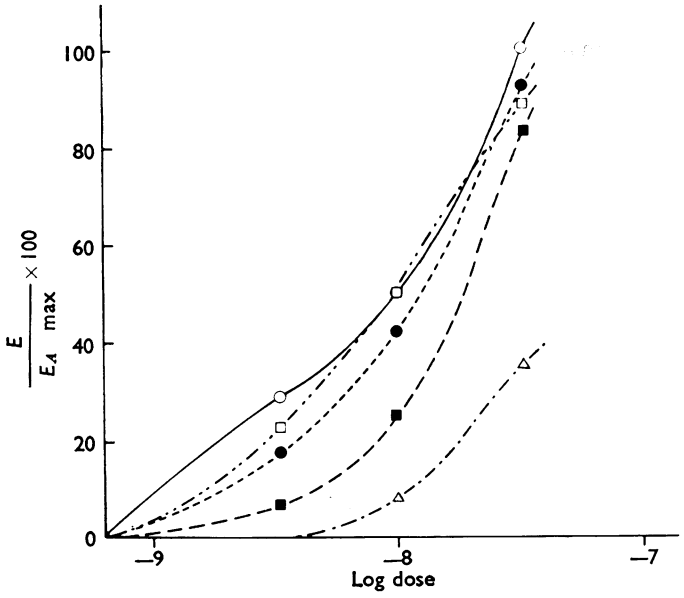


FIG. 2. Influence of fresh rat plasma on the anti-eledoisin activity of morin on the isolated guinea-pig ileum. Abscissa, log dose eledoisin; ordinate, $E/E_{A\max}$ where E is the effect produced by a given concentration of eledoisin and $E_{A\max}$ is the maximum effect produced by eledoisin. ○——○, Normal; ■– –■, morin 2 μg/ml.; △·—·△, morin 4 μg/ml.; □··—··□, morin 2 μg/ml.+fresh plasma (4 ml./l.); ●- - -●, morin 4 μg/ml.+fresh plasma (4 ml./l.).

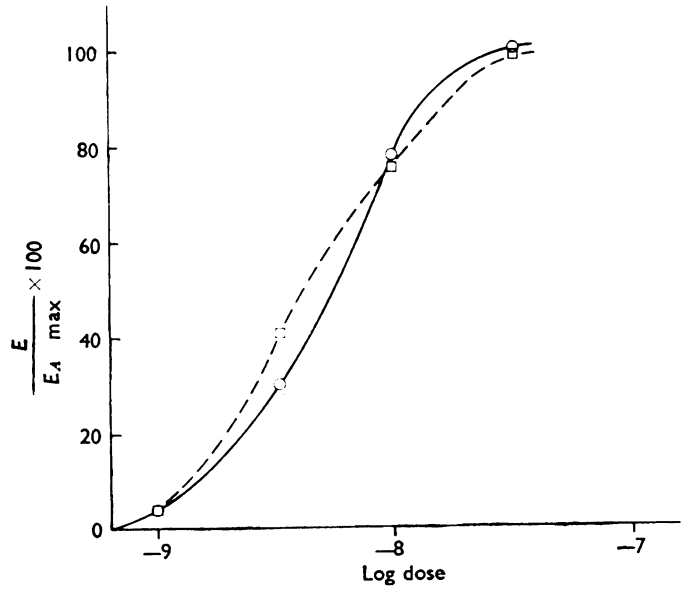


FIG. 3. Influence of deproteinized plasma on the anti-eledoisin activity of morin on the isolated guinea-pig ileum. Abscissa, log dose eledoisin; ordinate, $E/E_{A\max}$ where E is the effect produced by a given concentration of eledoisin and $E_{A\max}$ is the maximum effect produced by eledoisin. ○——○, Normal; □- - -□, morin 4 μg/ml.+deproteinized plasma (4 ml./l.).

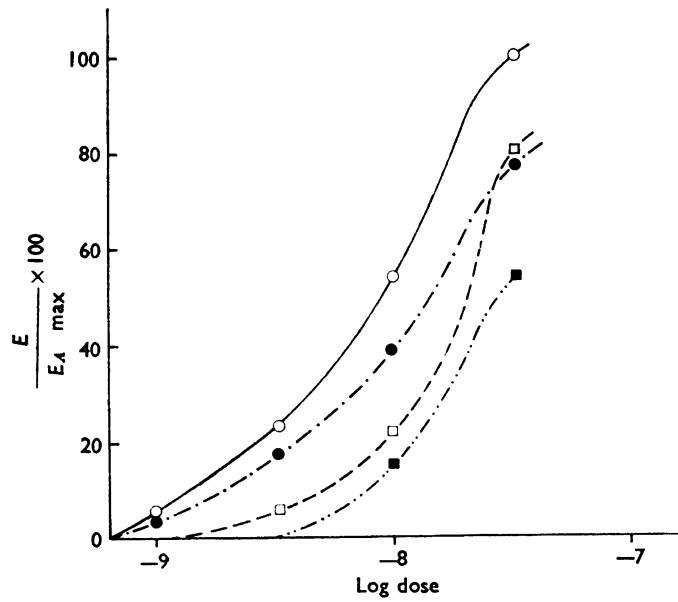


FIG. 4. Influence of deionized plasma on the anti-eledoisin activity of morin on the isolated guinea-pig ileum. Abscissa, log dose eledoisin; ordinate, $E/E_{A\max}$ where E is the effect produced by a given concentration of eledoisin and $E_{A\max}$ is the maximum effect produced by eledoisin. ○——○, Normal; □- - -□, morin 2 μg/ml.+deionized plasma (4 ml./l.); ■·—·■, morin 4 μg/ml.+deionized plasma (4 ml./l.); ●- · -●, morin 4 μg/ml.+deionized plasma (4 ml./l.)+cupric acetate 6×10^{-6}M.

We next studied the ability of various cations to restore the inhibitory effect of deionized plasma, using the acetate salts of the following cations: copper, cadmium, cobalt, zinc, strontium, nickel, aluminium and lead. Among these salts only cupric acetate could restore the inhibitory action of the deionized plasma (Fig. 4).

In another series of experiments, we studied the influence of cupric acetate and also the influence of a mixture of cupric acetate and N-acetyl-(±)-penicillamine (4 μg/ml.) on the inhibition induced by morin. Figure 5 shows the responses of a piece of guinea-pig ileum to eledoisin, the inhibitory effects of morin, the reversal of the effects of morin by cupric acetate and the failure to reverse the effects of morin when cupric acetate is added together with N-acetyl-(±)-penicillamine.

If the inactivation of morin by rat plasma is due to the formation of a complex between morin and the copper ions in the plasma, N-acetyl-(±)-penicillamine should

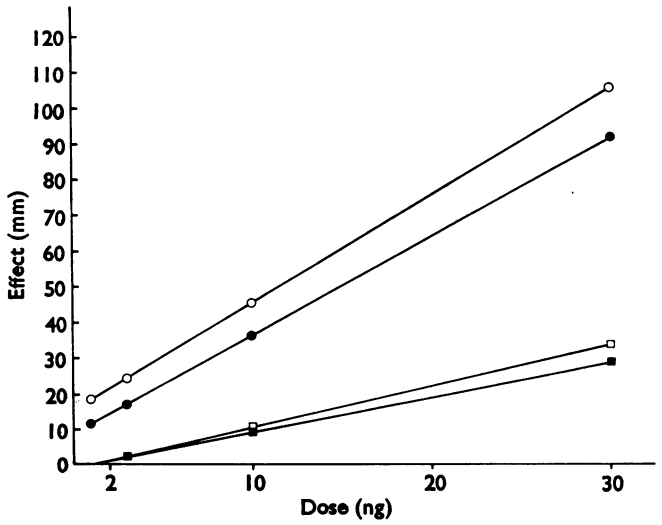


FIG. 5. Responses of a piece of guinea-pig ileum to eledoisin; influence of cupric acetate and N-acetyl-(±)-penicillamine on the inhibiting effects of morin. Abscissa, log dose eledoisin; ordinate, size of record of contraction (mm). ○——○, normal; □——□, morin 4 μg/ml.; ●——●, morin 4 μg/ml.+cupric acetate 6×10^{-6}M; ■——■, morin 4 μg/ml.+cupric acetate 6×10^{-6}M+N-acetyl-(±)-penicillamine (4 μg/ml.).

Statistical analysis

A: correlation coefficient

	r	*P*
Normal	0.8839	<0.001
Morin	0.8768	<0.001
Morin+cupric acetate	0.8918	<0.001
Morin+cupric acetate+N-acetyl-(±)-penicillamine	0.6965	<0.001

B: Student *t* test applied on the slope of the regression curves

	t	*P*
Normal *vs.* morin	6.4257	<0.001
Normal *vs.* morin+cupric acetate	0.8972	N.S.
Normal *vs.* morin+cupric acetate+N-acetyl-(±)-penicillamine	7.0418	<0.001
Morin+cupric acetate *vs.* morin	5.5285	<0.001
Morin+cupric acetate *vs.* morin+cupric acetate+N-acetyl-(±)-penicillamine	6.1445	<0.001
Morin *vs.* morin+cupric acetate+N-acetyl-(±)-penicillamine	0.6160	N.S.

be able to abolish this inactivation. The results in Fig. 6 are in agreement with this hypothesis: when morin was mixed with fresh rat plasma, the musculotropic activity of eledoisin was preserved. When the rat plasma was mixed with N-acetyl-(±)-penicillamine (4 μg/ml.) 30 min before its addition to the perfusion fluid, however, the anti-eledoisin activity of morin could still be elicited.

In the last series of experiments, we studied the influence of plasma obtained from rats pretreated with N-acetyl-(±)-penicillamine (4 mg/day per rat, i.p.) for periods of 5, 10 and 15 days on the anti-eledoisin activity of morin. The results in Fig. 7 indicate that after 5 days of pretreatment, there was still a reduction in the inhibitory effect of morin. There was no significant difference between the normal regression curve and the curve for morin plasma (5 days) or between the curves obtained with morin and morin + plasma, but the plasma no longer significantly modified the inhibitory power of morin after 10 days (Fig. 8) or after 15 days of pretreatment (Fig. 9).

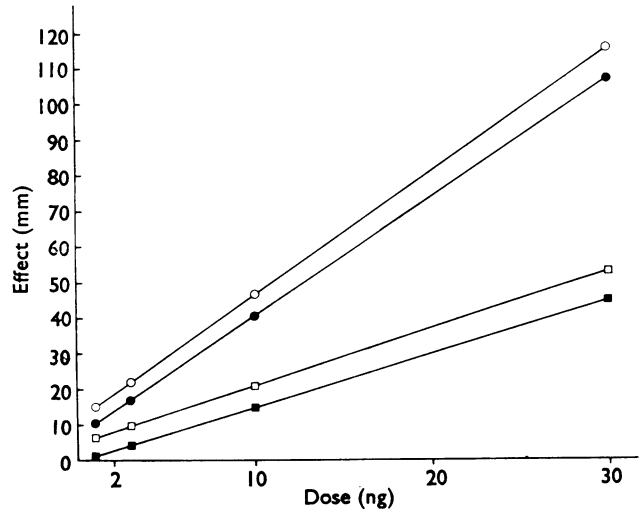


FIG. 6. Responses of a piece of guinea-pig ileum to eledoisin; influence of fresh plasma and N-acetyl-(±)-penicillamine on the inhibiting effects of morin. Abscissa, log dose eledoisin; ordinate, size of record of contraction (mm). ○——○, Normal; ●——●, morin 4 μg/ml. + fresh plasma (4 ml./l.); □——□, morin 4 μg/ml. + fresh plasma (4 ml./l.) + N-acetyl-(±)-penicillamine (4 μg/ml.); ■——■, morin 4 μg/ml.

Statistical analysis

A: correlation coefficient

	r	*P*
Normal	0.9009	<0.001
Morin	0.8596	<0.001
Morin + fresh plasma	0.9249	<0.001
Morin + fresh plasma + N-acetyl-(±)-penicillamine	0.8040	<0.001

B: Student *t* test applied on the slope of the regression curves

	t	*P*
Normal *vs.* morin	11.7634	<0.001
Normal *vs.* morin + fresh plasma	0.7413	N.S.
Normal *vs.* morin + fresh plasma + N-acetyl-(±)-penicillamine	11.1965	<0.001
Morin *vs.* morin + fresh plasma	11.0221	<0.001
Morin + fresh plasma *vs.* morin + fresh plasma + N-acetyl-(±)-penicillamine	10.4551	<0.001
Morin *vs.* morin + fresh plasma + N-acetyl-(±)-penicillamine	0.5669	N.S.

Rat blood pressure

The results observed on rat blood pressure were similar to those reported previously (Gascon, 1968a); that is, the hypotensive effect of eledoisin could not be modified by morin. Furthermore, in a series of ten experiments we also studied the influence of the flavonol derivative in rats pretreated 15 days with N-acetyl-(±)-penicillamine. Even in the pretreated animals, morin failed to antagonize the action of eledoisin.

Discussion

The results obtained here confirm our previous findings (Gascon, 1968a) that eledoisin antagonizes the musculotropic activity of eledoisin on the isolated guinea-pig ileum. In addition, we observed that, when fresh rat plasma or deproteinized rat plasma is added to the Tyrode solution before the addition of morin, the latter completely loses its anti-eledoisin activity, indicating that both types of plasma have the ability to inactivate the flavonoid derivative and that the plasma proteins do

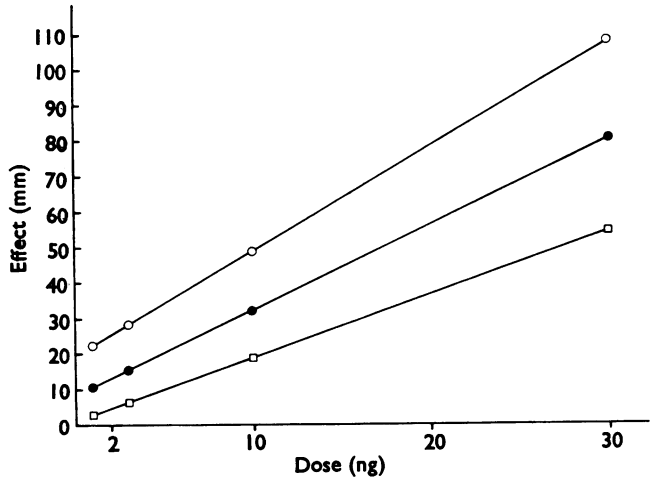


FIG. 7. Responses of a piece of guinea-pig ileum to eledoisin; influence of plasma from animals pretreated with N-acetyl-(±)-penicillamine (4 mg/rat per day for five days) on the inhibiting effects of morin. Abscissa, log dose eledoisin; ordinate, size of record of contraction (mm). ○——○, Normal; ●——●, morin 4 μg/ml. + plasma (4 ml./l.); □——□, morin 4 μg/ml.

Statistical analysis

A: correlation coefficient

	r	*P*
Normal	0.6943	<0.01
Morin	0.7559	<0.01
Morin + plasma	0.6715	<0.02

B: Student *t* test applied on the slope of the regression curves

	t	*P*
Normal *vs.* morin	2.8979	<0.01
Normal *vs.* morin + plasma	1.3380	N.S.
Morin *vs.* morin + plasma	1.5598	N.S.

not seem to play a part in this inactivation. On the basis of its structure (Fig. 1), it could be thought that morin would be a potent chelator, a property that has been reported for most of the flavonoids (Clark & Geissman, 1949) and also that part of its inactivation may be the result of the formation of a complex with some plasma cations.

The fact that the deionized plasma does not abolish the inhibitory effect of morin supports the idea that formation of a complex between morin and some plasma cation occurs. In this connection, addition of cupric acetate to the deionized plasma restored the ability of the deionized plasma to inactivate morin, but all the other cations studied were inactive even at concentrations much higher than their respective content in rat plasma.

From these results, we can conclude that the inactivation of morin by the rat plasma was due to the formation of a complex with the plasma copper. This conclusion is supported by the fact that the effect of fresh rat plasma as well as that of cupric acetate is abolished by (−)-penicillamine and N-acetyl-(±)-penicillamine, two copper chelators (Goodman & Gilman, 1965). Another argument in favour

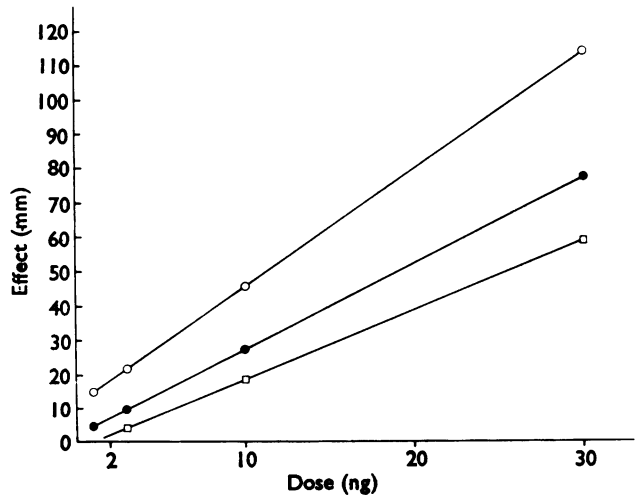


FIG. 8. Responses of a piece of guinea-pig ileum to eledoisin; influence of plasma from animals pretreated with N-acetyl-(±)-penicillamine (4 mg/rat per day for 10 days) on the inhibiting effects of morin. Abscissa, log dose eledoisin; ordinate, size of record of contraction (mm). ○——○, Normal; ●——●, morin 4 μg/ml.+plasma (4 ml./l.); □——□, morin (4 μg/ml.).

Statistical analysis

A: correlation coefficient

	r	*P*
Normal	0.8609	<0.001
Morin	0.8130	<0.001
Morin+plasma	0.8361	<0.001

B: Student *t* test applied on the slope of the regression curves

	t	*P*
Normal *vs.* morin	4.1551	<0.001
Normal *vs.* morin+plasma	2.7972	<0.02
Morin *vs.* morin+plasma	1.3578	N.S.

of this hypothesis is that the plasma of animals treated for 10 to 15 days with N-acetyl-(±)-penicillamine did not reduce the anti-eledoisin activity of morin.

The interaction between morin and the plasma copper does not, however, explain the lack of effect of morin on the rat blood pressure, because the eledoisin inhibitor is still inactive in animals previously treated with N-acetyl-(±)-penicillamine for 15 days. The absence of anti-eledoisin activity of morin on the blood pressure may be the result of an interaction between the flavonoid and the copper cations in other tissues. Another possibility is the rapid biotransformation of morin by some enzymatic system.

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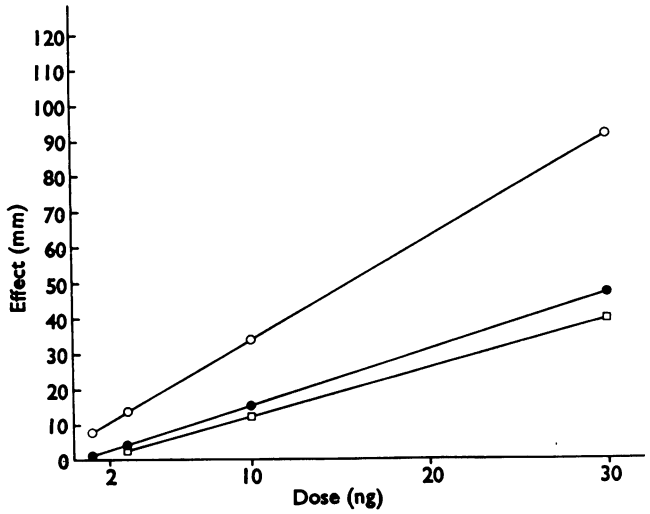

FIG. 9. Responses of a piece of guinea-pig ileum to eledoisin; influence of plasma from animals pretreated with N-acetyl-(±)-penicillamine (4 mg/rat per day for 15 days) on the inhibiting effects of morin. Abscissa, log dose eledoisin; ordinate, size of record of contraction (mm). ○——○, Normal; ●——●, morin 4 μg/ml.+plasma (4 ml./l.); □——□, morin 4 μg/ml.

Statistical analysis

A: correlation coefficient

	r	*P*
Normal	0.9213	<0.001
Morin	0.8592	<0.001
Morin+plasma	0.8911	<0.001

B: Student *t* test applied on the slope of the regression curves

	t	*P*
Normal *vs.* morin	4.4639	<0.001
Normal *vs.* morin+plasma	3.8846	<0.001
Morin *vs.* morin+plasma	0.5792	N.S.

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