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ON THE MECHANISM OF ACTION OF COLISAN

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Purified Colisan produces instantaneous lysis of paramecia and amoebae, and blebbing of paramecia, rat mast cells and ascites tumour cells. Colisan liberates histamine from mast cells. It also suppresses competitively the bradykinin-provoked contraction of the guinea-pig ileum. Most of these effects are not shared by staphylococcus-haemolysin. The results suggest a rapid change of the permeability of the cell membrane as the mechanism of all the diversified biological actions of Colisan.

In previous publications the dual effect of Colisan, the active principle of the bacterial mutant RB-103, was described (Bergmann, Reitler, Chaimovitz & Bergmann, 1960; Bergmann, Leon, Chaimovitz & Benzakein, 1961). On the one hand, this extract produces lysis of single cells such as protozoa or erythrocytes; on the other, it counteracts smooth muscle stimulants. Furthermore, it was shown that staphylococcus β-haemolysin, although differing in certain respects, displays a similar combination of biological effects. With further progress in the purification of the active material, new evidence was adduced for the direct amoebicidal action of Colisan, for which unambiguous proof had not been available previously. Likewise it became possible to define more precisely the mechanism of action of the antibiotic by studying the immediate effect of concentrated solutions on isolated cells. These experiments also brought to light new characteristic differences between β-haemolysin and Colisan.

METHODS

β-Haemolysin was the commercial preparation of the Wellcome Research Laboratories, Beckenham, England, containing 0.08 i.u./mg. Bradykinin was a gift of the Sandoz Corporation, Basle. Colisan, obtained from Hillel Remedies, Haifa, was purified by a chromatographic method to be described separately. The dry material used in the present experiments killed paramecia at a concentration of about 10 μg/ml., under the standard conditions described previously (Bergmann *et al.*, 1961). Crystalline paromomycin sulphate was a gift of Parke, Davis & Company, Hounslow, England.

Mast cells were obtained from white rats of about 100 g body weight by the following procedure. Two intraperitoneal injections of 10 ml. of 0.9% sodium chloride solution were administered within an interval of 2 min. The animal was wrapped in a towel and shaken gently. About 2 min after the second injection a turbid fluid was withdrawn from the peritoneal cavity. The cells in this fluid were spun down, washed with saline and used at once for the experiment.

Morphological changes of the mast cells were photographed with the aid of a Wild phase-contrast microscope, put at our disposal by Dr. A. Bekierkunst, of the Department of Bacteriology of this Medical School.

Ascites tumour cells of the Landschütz strain were obtained from Dr L. Bloch-Frankenthal, Department of Experimental Medicine.

Tests on guinea-pig ileum were performed as described earlier (Bergmann *et al.*, 1960).

RESULTS

Immediate effect of Colisan on amoebae. When purified Colisan was added to a suspension of *Entamoeba histolytica,* in concentrations 10 times higher than those used for 48 hr tests (approximately 1 mg/ml.), the following immediate changes could be observed under the microscope: (*a*) The amoebae ceased to send out

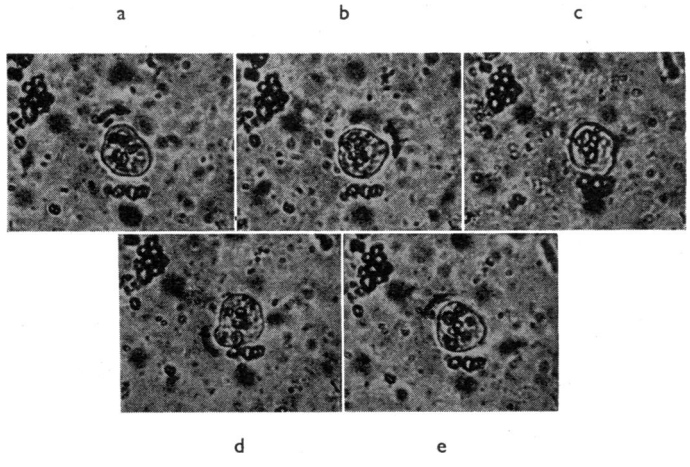

Fig. 1. Effect of Colisan on *Entamoeba histolytica.* In the centre of the picture a single cell is shown. Bulge rotating clockwise around the surface of the cell indicated by arrow. Small particles inside and below cell are starch granules. Pictures taken with film camera, magnification ×400.

pseudopodia and assumed a rounded form. (*b*) A small protuberance bulged from the surface and started travelling around the cell at considerable speed (Fig. 1*a–e*). (*c*) This movement stopped suddenly and a few seconds later the cell burst, voiding its content in one direction and leaving only a ghost (Fig. 2*a–d*).

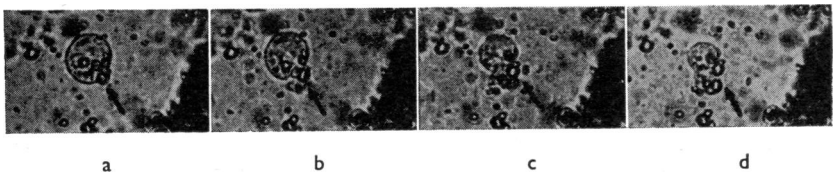

Fig. 2. Lysis of *Entamoeba histolytica* under the influence of Colisan. Four stages (*a–d*) of the process of shedding the starch granules inside the cell are shown. Arrow points to opening in the cell surface. (Under the phase microscope, ejection of the nucleus could also be observed.) Picture taken with film camera, magnification ×400.

The whole process required 1 to 3 min, depending on the Colisan concentration.

No immediate action on amoebae could be detected by application of β-haemolysin in a concentration of 1 i.u./ml., that is, about 25 times higher than the concentration active in 48 hr tests (Bergmann *et al.*, 1961). Paromomycin (10 mg/ml.) immobilized the amoebae within about 30 min, without, however, causing lysis.

Instantaneous action of Colisan on Paramecium caudatum. With these protozoa, the following rapid changes were observed, when Colisan concentrations, 2 to 5 times higher than those effective in a 24 hr test (20 to 50 μg/ml.), were added to the medium: (*a*) The protozoa halt their propulsion and turn around in narrow circles. (*b*) The paramecia repose on the same spot, but rotate around their longitudinal axis. Blisters sprout from various parts of the surface, but usually not from the poles (Fig. 3*b*), and rapidly increase in size, so that the cell volume

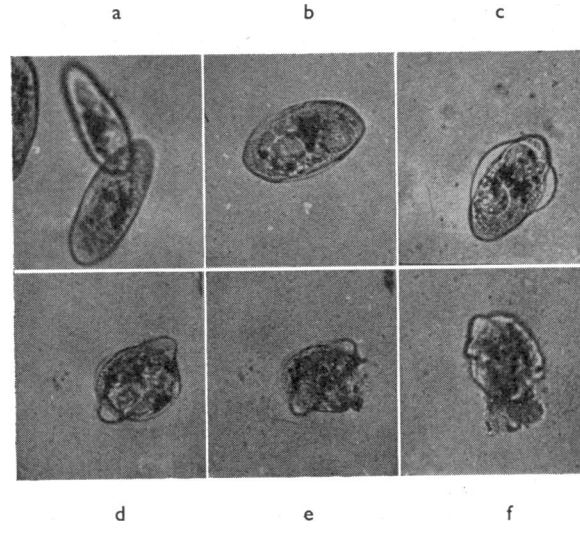

Fig. 3. Rapid morphological changes of *Paramecium caudatum* under the influence of Colisan. (*a*) Normal cells, in rapid propulsion. (*b*) After addition of 50 μg/ml. of Colisan, cell moving in narrow circle. Initial stage of blister formation along both flanks. (*c*) Blisters have increased in size and are sharply separated from cell body. The protozoon moves forward very slowly. (*d*) Locomotion stopped. Paramecium rotates around its longitudinal axis. Enormous swelling of cell body. The bulge to the right indicates the spot at which cell will burst in the next moment. (*e*) Incipient eruption. (*f*) Eruption completed. Only a deformed shadow of the cell is left. Pictures taken with film camera, magnification ×400.

may be expanded by 100% or more (Fig. 3*c*). In general, the large vesicles are transparent and separated from the main cell body by a fine demarcation line (Figs. 3*c* and 4*a*). Finally, rotation ceases. In rare cases the bleb was not separated from the cell body, as evidenced by the fact that it contained numerous granular particles (Fig. 4*b* and *c*). Some of the bizarre forms that can be observed at this stage are shown in Fig. 4. (*c*) One of the blisters bursts and the cell contents are ejected, to leave behind only a deformed ghost (Fig. 3*d–f*). The whole process requires 30 to

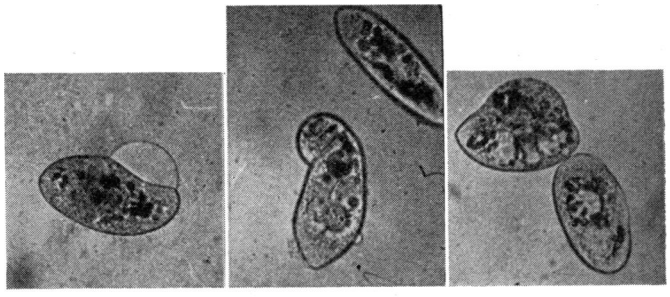

Fig. 4. Some peculiar shapes of paramecia, appearing after treatment with high concentrations of Colisan. (*a*) Eccentric bleb, perfectly transparent and demarcated against main cell body by a sharp border. Such asymmetrical blisters, when sufficiently large, prevent rotation of the protozoon around its longitudinal axis. (*b*) Upper—normal paramecium; lower—blister sprouting from upper pole in open connexion with cell body and therefore filled with granules. (*c*) Upper cell shows bleb protruding from the flank, but remaining in connexion with main cell body. Picture taken with film camera, magnification ×400.

90 sec. With still higher concentrations of Colisan, it may take only a few seconds—the time needed to add the antibiotic to the medium—to blot out all paramecia. Again, no immediate action of β-haemolysin on paramecia could be detected. With concentrations of 1 i.u./ml., the protozoa kept moving freely for at least 1 hr. Similar observations were made with paromomycin (10 mg/ml.).

Influence of tonicity of the medium on the reaction of paramecia to Colisan. The fact that, under the influence of Colisan, paramecia increase their volume considerably, while amoebae undergo lysis without measurable swelling, may be ascribed to the different tonicity of the media used. The paramecia are raised and examined in tap water. They can adapt themselves to varying osmotic concentrations of the milieu by adjusting the rate of pulsation of the contractile vacuoles (Wichtermann, 1953). Indeed, in 0.5 molar salt solutions, the protozoa survive the effect of " lethal " concentrations of the antibiotic for much longer periods (up to 10 min) forming—if at all—only small blebs. Finally, however, the paramecia undergo lysis even without previous blister formation.

Effect of Colisan on red blood corpuscles. Rabbit erythrocytes, when incubated with Colisan concentrations, about 10 times higher than those used in haemolysis tests for determination of H_{50} (Bergmann *et al.*, 1961), did not show any sudden morphological changes. They faded gradually and disappeared finally, giving the impression that haemoglobin was released from the cells within a short time.

Response of mast cells to Colisan. Mast cells, suspended in 0.9% sodium chloride solution, underwent a series of characteristic changes: (*a*) Transparent blebs were formed, covering often the whole surface and being surrounded by a well-defined membrane (Fig. 5*b*). While the blister increased in size, the nucleus remained invisible (Fig. 5*c*). (*b*) After exposure to higher concentrations of Colisan, the nucleus became visible (Fig. 5*e* and *f*), apparently because of changes in the ratio of the refractive indices of nuclear and protoplasmatic masses. The nucleus

was delimited by a ring of dense (black) granules, and in some cases it was found in an eccentric position, giving the impression of being extruded from the cell. A single, eccentric bleb may be formed (Fig. 5*c*) or two or more blisters may protrude from different parts of the surface (Fig. 5*d*). Ultimately, all protuberances coalesce (Fig. 5*e*).

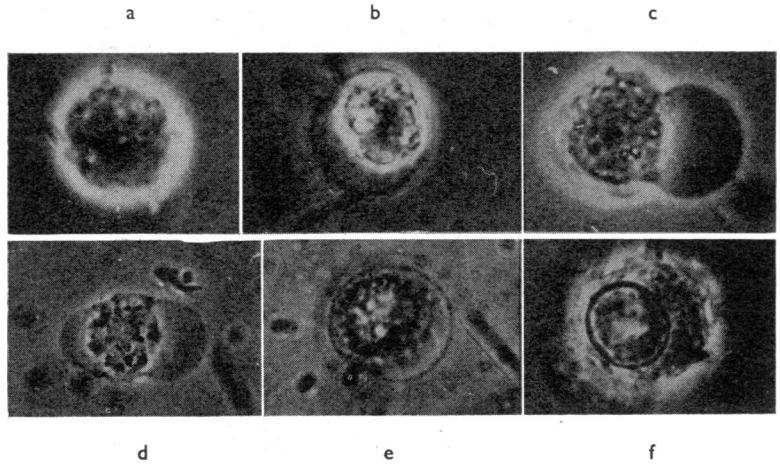

Fig. 5. Morphological changes of rat mast cells under the influence of purified Colisan. (*a*) Untreated mast cells. (*b*) Small concentration of the antibiotic. Note incipient *unilateral* bleb. (*c*) Same concentration of Colisan as in (*b*), but later stage. Large unilateral bleb. Cell granules clearly discernible. (*d*) Same as in (*c*), another cell with *bilateral* bleb formation. (*e*) Higher concentration of Colisan. Dark granules, crowded around a central area (supposedly the cell nucleus). (*f*) Same concentration as (*e*). Note sharply defined nucleus; no granules visible in protoplasm. Photographs by phase-contrast microscope, magnification ×400.

The swiftness with which these changes took place indicated a direct action of Colisan on the cell membrane, altering its permeability. It was thus suspected that the drug may act as histamine releaser. For such tests, it is essential to apply only small concentrations of Colisan, which do not produce morphological changes beyond the stage of Fig. 5*b*, and which—under the conditions of the intestinal test—are insufficient to antagonize the histamine-induced contractions, an effect of Colisan described previously (Bergmann *et al.*, 1960). By selection of the proper dose of Colisan, it was indeed possible to separate the undamaged mast cells, after 15 min incubation, from the medium and to test the supernatant on guinea-pig ileum. As shown in Fig. 6, a very strong contraction resulted, which was abolished by previous treatment of the gut with diphenhydramine hydrochloride. Only insignificant amounts of histamine were released from rat mast cells when incubated for the same length of time with 0.9% sodium chloride solution alone (Fig. 6*c*).

The analogous experiment with β-haemolysin was not feasible. Small concentrations of this material did not liberate histamine from the mast cells. Higher concentrations blocked the action of histamine on the ileum, so that the release of the compound from mast cells could not be tested. Heating to 60° for 30 min

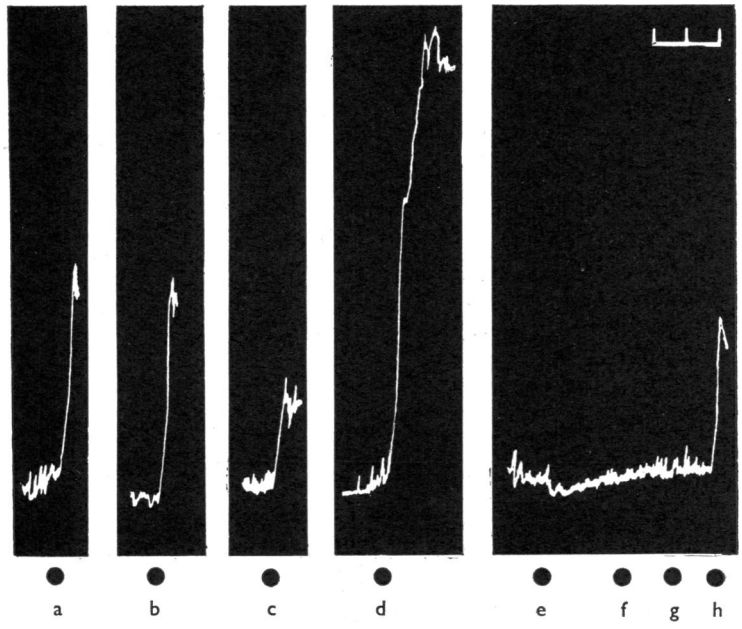

Fig. 6. Demonstration of Colisan-induced histamine release from rat mast cells. (*a*) 6 ng/ml. acetylcholine; (*b*) 6 ng/ml. histamine; (*c*) 1 ml. of supernatant of mast cells, incubated with 3 ml. of saline; (*d*) 0.2 ml. of supernatant of mast cells, incubated with 100 μg/ml. of Colisan powder (final concentration of Colisan in organ bath: 0.4 μg/ml.); (*e*) 0.08 μg/ml. diphenhydramine hydrochloride; (*f*) 3 min later, without washing: same as (*d*); (*g*) 6 ng/ml. histamine; (*h*) 6 ng/ml. acetylcholine.

abolishes the haemolytic effect of α-toxin (Robinson, Thatcher & Montford, 1960), while heating to 100° for 10 min abolishes the action of this compound upon the intestinal muscle (Brown, Prichard and Quilliam, 1959). However, the antagonistic action of β-toxin on the smooth muscle was not impaired by either procedure. The morphological alterations of ascites tumour cells under the influence of Colisan were essentially identical with the changes described for mast cells.

Interaction of Colisan with bradykinin. The smooth muscle contraction, induced by bradykinin, is distinguished from the effect of other stimulants, such as acetylcholine or histamine, by its slow onset and gradual decay. That the mechanism of bradykinin contraction differs essentially from that of other stimulants is evident from the observation that an intestinal loop, which has just recovered from a bradykinin-provoked contraction, still gives full response to acetylcholine (Fig. 7*b* and *c*). It is also well known that bradykinin is not antagonized appreciably by atropine or antihistamines (Rocha e Silva, 1955; Konzett & Stürmer, 1960). As Colisan elicits a slow but lasting relaxation of the smooth muscle, it may be a suitable antagonist of bradykinin. Figs. 7 and 8 demonstrate partial and complete suppression of the latter's activity by previous incubation of the gut with the antibiotic. This antagonism is of competitive character, as 10 times higher doses of bradykinin overcame the Colisan block (Fig. 8*d*). It is of interest that maximal concentrations of β-haemolysin (0.4 i.u./ml.) had no inhibitory effect on bradykinin-induced contractions. It

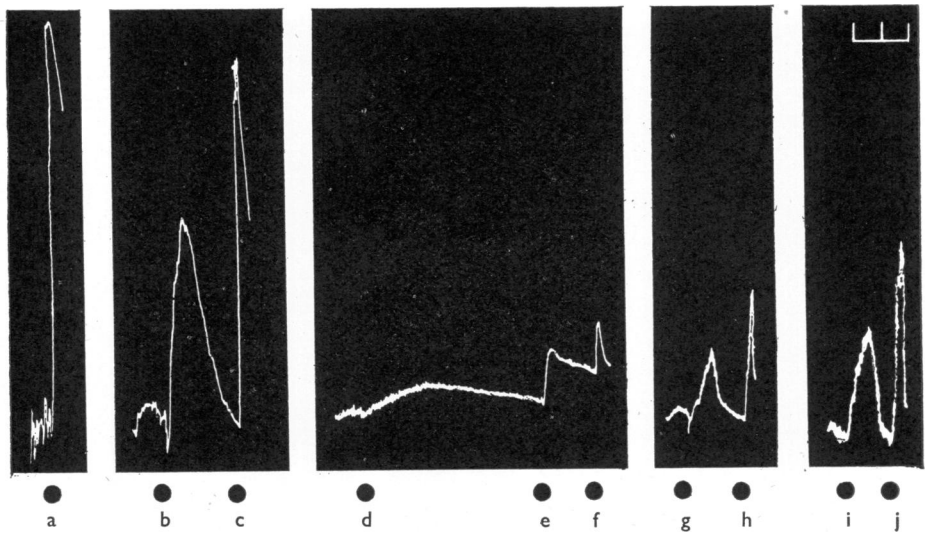

Fig. 7. Reversible suppression of bradykinin-provoked contraction of guinea-pig ileum by Colisan. Fresh ileum; acetylcholine dosage, 4 ng/ml.; bradykinin dosage, 40 ng/ml. (*a*) Acetylcholine; (*b*) bradykinin; (*c*) 3 min later, without washing, addition of acetylcholine; note almost undiminished height of contraction; (*d*) 20 μg/ml. Colisan; (*e*) after 7 min incubation, without washing, addition of bradykinin; (*f*) 2 min later, without washing, addition of acetylcholine; between (*f*) and (*g*), 3 washings during 10 min; (*g*) bradykinin; (*h*) without washing, acetylcholine; between (*h*) and (*i*), 3 washings during 10 min; (*i*) bradykinin; (*j*) acetylcholine.

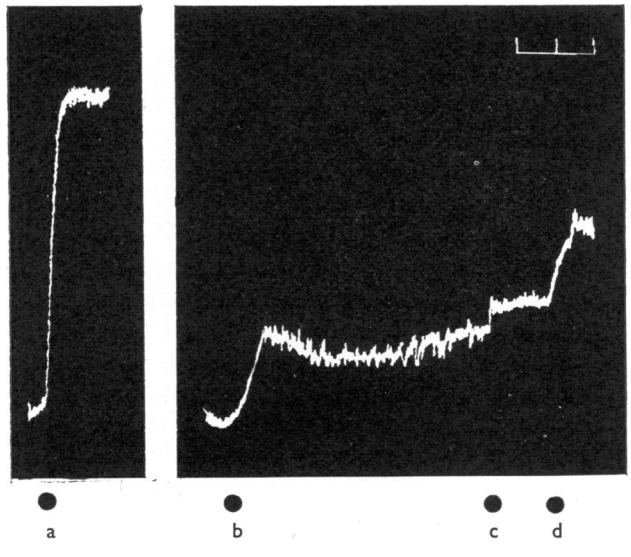

Fig. 8. Competitive character of the Colisan-bradykinin antagonism. Fresh guinea-pig ileum. (*a*) 60 ng/ml. bradykinin; (*b*) 40 μg/ml. Colisan powder; (*c*) after 7 min, without washing, addition of bradykinin, 60 ng/ml.; practically complete suppression of its effect; (*d*) 2 min later, without washing, 600 ng/ml. bradykinin. Contraction height about 1/4 of standard contraction at (*a*).

has been reported previously (Bergmann *et al.*, 1961) that 1/5 of this concentration abolishes the response to acetylcholine, histamine, 5-hydroxytryptamine and other smooth muscle stimulants.

Inability of bradykinin to antagonize the lytic effects of Colisan. The interference of Colisan with bradykinin-provoked contractions of the ileum suggested a similar antagonism of the two compounds towards isolated cells. However, bradykinin did not protect paramecia, amoebae or rabbit erythrocytes against the action of Colisan. On the other hand, bradykinin exerts a weak effect of its own against paramecia. The maximal concentrations available to us (100 μg/ml.) were, however, inert towards *Entamoeba histolytica.*

DISCUSSION

The present experiments support the assumption that Colisan acts directly on protozoal cells. For example, lysis of amoebae by a purified extract of the antibiotic proceeds within a few minutes, so that involvement of symbiotic bacteria can be excluded. The rapid formation of blisters in paramecia or tissue cells is an expression of a sudden change of permeability, enabling water to penetrate quickly into the membrane phase. Usually the membrane separates into two layers, so that the bleb remains free of protoplasmatic granules. In this case, solutes and/or colloids must diffuse from the cell interior to the blister in order to explain its steady growth. Occasionally, however, the swelling process involves the cell body itself (as, for example, in Fig. 4*b* and *c*).

The morphological changes of single cells, observed under the influence of Colisan, are not exceptional. Bleb formation, either spontaneous (Zollinger, 1948) or induced by cytotoxic agents (Belkin & Hardy, 1961), has been reported for normal and malignant cells. As a rule, these processes are much slower than the formation of blisters under the action of Colisan. However, Easty & Ambrose (1957) obtained lysis of mouse ascites tumour cells by rabbit immune serum within a few minutes.

It appears significant that amoebae do not undergo analogous changes before Colisan-induced lysis. This disparity is related to the different function and structure of the cell membrane. The amoeba perpetually suffers large changes of its surface area, requiring either shifting or neogenesis of building material (Bell, 1961). Although these continuous changes may alter the membrane permeability, the cell nevertheless does not undergo marked volume changes. It appears that amoebae are less prone to swelling than other protozoa, which dispose of a denser and structurally better defined surface pellicle.

The effect of Colisan on protozoal cells is characteristically distinguished from the action of antibodies. Thus, paramecia are immobilized by rabbit antiserum, because under this treatment they secrete a polymeric material that sticks to their outer surface and causes agglutination (Bernheimer & Harrison, 1940). In the presence of complement, lysis will also ensue. Similarly, antisera against amoebae produce rounding-up, immobilization and lysis (Zaman, 1960), but without the characteristic changes, described in Fig. 1 of the present study.

The direct surface action of Colisan is also supported by the (unpublished) observation that the active principle diffuses only slowly through a cellophane

membrane. Therefore, within the short time-span available for its lytic effect, Colisan could hardly reach the interior of the cell to interfere with its biochemical apparatus. If such a mechanism should be involved in the biological action of Colisan, it must be sought near the outer surface. Further progress in the understanding of the permeability changes involved requires more knowledge about the chemical structure of the antibiotic.

The suppression by Colisan of the bradykinin-induced contraction of the intestine corroborates our previous statement that the action of the antibiotic on the smooth muscle cell is direct and unspecific. Bradykinin, on the other hand, appears to require a specific receptor, as its antagonism to Colisan does not extend to other systems such as isolated cells undergoing lysis.

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