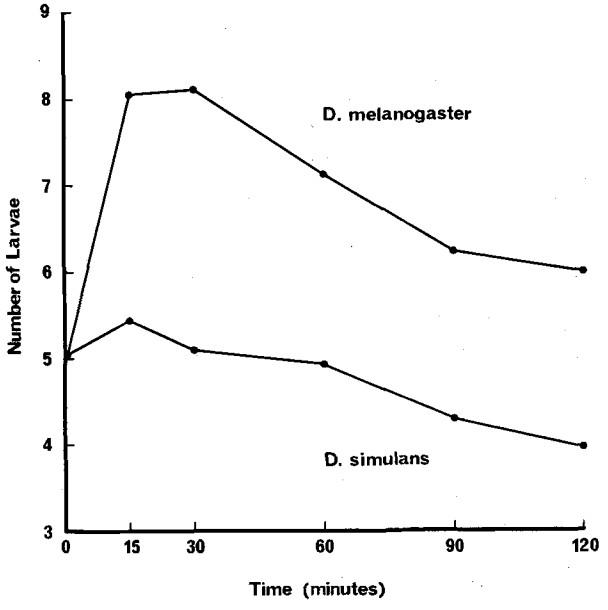
equivocal than oviposition data[6], suggesting a much higher level of specificity for larval behaviour. This is consistent with the exploitation of a variety of foods by a cosmopolitan species since, following hatching, larvae can move to the most beneficial resources available to them. However, in many rare endemic species, quite specific oviposition stimuli presumably occur because of highly specific larval resource exploitation, often involving parts of particular plant species[7].

The known ecological differences between these 2 species are principally quantitative rather than qualitative, both species apparently using rather similar resources[8] with the single exception of ethanol. However, since studies concentrating on larvae are rare, future research could alter this situation. The comparative study of resource utilization by larvae of different Drosophila species remains an open field, and detailed investigations in particular of sibling species may yield interesting information concerning evolutionary divergence.



Mean number of larvae out of 10 on ethanol containing agar up to 120 min for the 2 sibling species.

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2 J. A. McKenzie and P. A. Parsons, Oecologia *10*, 373 (1972).
3 J. A. McKenzie, Oecologia *15*, 1 (1974).
4 D. Sewell, B. Burnet and K. Connolly, Genet. Res. *24*, 163 (1975).
5 M. Libion-Mannaert, J. Delcour, M. C. Deltcombe-Lietaert, N. Lenelle-Montfort and A. Elens, Experientia *32*, 22 (1976).
6 S. King, R. F. Rockwell and J. Grossfield, Genetics *83*, 539 (1976).
7 H. L. Carson, D. E. Hardy, H. T. Spieth and W. S. Stone, in: Essays in Evolution and Genetics in Honor of Theodosius Dobzhansky, p. 437. Ed. M. K. Hecht and W. C. Steere. Appleton-Century-Crofts, New York 1970.
8 P. A. Parsons, Q. Rev. Biol. *50*, 151 (1975).

Adenylate cyclase activation by trypsin in KB cell cultures

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Summary. Trypsin commonly used for cell dispersion increases adenylate cyclase activity of KB cells. It acts on catalytic receptors, since the apparent K_m for ATP is lowered, and it alters the dependence of adenylate cyclase on Mg^{++} ions.

Evidence has accumulated suggesting that cyclic AMP can influence cell divisions in a number of cell types in culture[1,2]. Certains agents, such as pronase, which are known to act on the cell surface, can initiate cell divisions. Bombick and Burger[3] have shown that the action of such agents can be blocked by the addition of dibutyryl cyclic AMP. It seems that extracellular conditions may control cell divisions by affecting the membranal components of the cyclic AMP system, i.e. the adenylate cyclase. This paper describes the effects on adenylate cyclase of procedures commonly used to detach cells from flasks during cell cultures.

Materials and methods. KB cells were grown in 75 cm culture surface Falcon flasks containing 20 ml of Eagle's minimal essential supplemented with 10% calf serum, 0.25% sodium bicarbonate and 0.005% aureomycin (pH = 7.1). Approximately 8×10^6 cells were seeded from confluent cells dispersed by one of the 3 commonly used methods: scraping with glass taws, treatment with 0.25% trypsin for 2 min, or treatment with 2.5% ethylene diamine tetraacetic sodium salt (EDTA) for 10 min. Changes of media for the feeding of culture were made 42 h later. In these conditions, confluency is obtained 48 h later. For cell numerations, cell viability was routinely tested by eosine dye exclusion. For adenylate cyclase assay cells were harvested by scraping in 25 mM Tris-HCl pH = 7.6 supplemented with 1 mM $MgCl_2$ and 250 mM saccharose, broken at 4 °C and centrifugated at $600 \times g$ for 10 min, the pellet was routinely used for assays. 3 culture flasks were pooled for each enzyme activity determination. Assay reaction constituents[4] included 2 mM (α^{32}P)-ATP 1 μCi, 1 mg/ml creatine phosphokinase, 20 mM creatine phsophate, 1 mg/ml bovine serum albumine, 10 mM $MgCl_2$ and enzyme (approximately

1 J. Otten, G. Johnson and I. Pastan, Biochem. biophys. Res. Commun. *44*, 1192 (1971).
2 E. Rozengurt and A. Pardee, J. Cell Physiol. *80*, 273 (1972).
3 B. M. Bombik and M. M. Burger, Exp. Cell Res. *80*, 88 (1973).
4 A. Guiraud-Simplot and L. Colobert, C. r. Acad. Sci, Paris *283*, 93 (1976).

400 µg protein) in a final volume of 100 µl. Reactions were initiated by addition of enzyme at 4 °C, were incubated at 37 °C for 10 min in a shaking water-bath and were immersed in boiling water for 3 min. We added 100 µl stop solution containing 20 mM ATP and 6 mM (8^3H)-cyclic AMP (5000 cpm) as chromatographic tracer. The cAMP formed was isolated according to the method of White[5]. The recovery of the carrier (8^3H)-cyclic AMP varied from 50% to 60%. Immersion was carried out and stop solution was added prior to the addition of enzyme in blank reactions and blank values were subtracted from each experiment value. All enzyme assays were carried out in duplicate. Adenylate cyclase activities expressed as pmoles of cyclic AMP formed during the reaction were linear with respect to time of incubation up to 20 min and to protein concentration up to 1 mg protein. Activities were routinely expressed as pmoles of cyclic AMP formed per minute per mg protein (pmoles/min/mg prot.).

Results. Figure 1 shows increased adenylate cyclase activity following transfer of KB cells removed from culture flasks with trypsin. This effect lasts several hours. Scraping or EDTA, used instead of trypsin to remove cells, does not cause such activition. The numeration of KB cells does not show stimulation of cell divisions after trypsin treatment, in the used culture conditions. In order to characterize further the effect of trypsin on the stimulation of adenylate cyclase, the activities were determined in the presence of increasing ATP concentrations with constant $MgCl_2$ concentration (figure 2, A) and in the presence of increasing $MgCl_2$ concentrations with constant ATP concentration (figure 2, B). Adenylate cyclase of cells detached by trypsin shows an apparent K_m for ATP lower than that of cells detached by scraping or EDTA (K_m = 0.5 mM instead of K_m = 0.7 mM). The dependence of adenylate cyclase on Mg^{++} ions is also different. With the assay method we used, there is no significant activity in cells detached by scraping or EDTA when $MgCl_2$ concentration is lower than 2 mM, but we obtained 15% of maximal activity in cells detached by trypsin. This effect is notable because Mg^{++} concentration in culture medium is 2.5 mM.

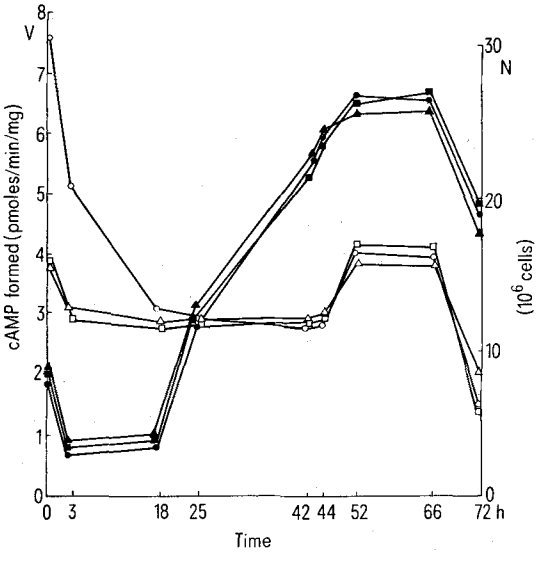

Fig. 1. This data shows the effect of the method used to disperse cells before seeding, on adenylate cyclase activity over a period of culture. Number of living cells (● ■ ▲), adenylate cyclase activity (○ □ △), cell dispersion by scraping (□ ■), EDTA (△ ▲), trypsin (○ ●).

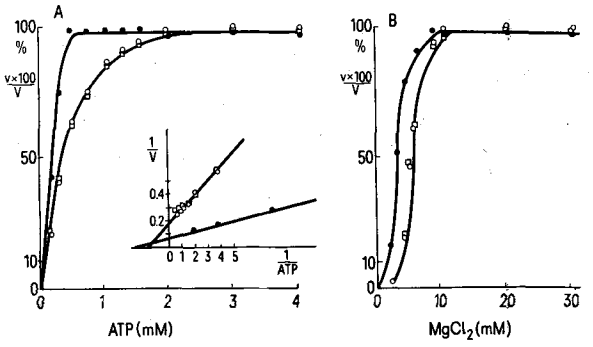

Fig. 2. Confluent cells from 10 culture flasks were pooled and adenylate cyclase activity was assayed in triplicate. *A* shows the relative activities as a function of ATP concentrations with 10 mM $MgCl_2$, the smallest panel shows the corresponding Lineweaver et Burk plots. *B* shows relative activities as a function of $MgCl_2$ concentrations with 2 mM ATP. Cell dispersion by scraping (□), trypsin (●), EDTA (○).

Discussion. Trypsin is known to decrease the activity of cyclic nucleotide phosphodiesterase located in plasma membrane[6], but also to cause a decrease in the cyclic AMP level[7] and stimulation of cell divisions[3,8]. There is a discrepancy between these findings and our results concerning the influence of trypsin on adenylate cyclase activity. Ryan et al.[9] suggest that cyclic AMP may be synthetized at a rapid rate but it may also be lost by the cell very quickly because trypsin produces increased permeability of cells[10,11]; therefore a decrease in cellular cyclic AMP may occur. In fact, the levels of cyclic AMP in culture medium should have been determined before and after trypsin treatment to confirm this hypothesis. Figure 1 shows that there is no stimulation of cell divisions after trypsin treatment, although such stimulation is generally attributed to the action of proteases on cells. This reaction might be different in special transformed cells such as KB cells, or might vary with time and according to the conditions of the trypsin treatment. Adenylate cyclase activity is enhanced by trypsin when used for only 2 min, therefore a new enzyme synthesis does not seem to be probable. Trypsin acts on the catalytic receptor since the apparent K_m for ATP is lowered (figure 2, A), from 0.7 to 0.5 mM, but the real substrate of this enzyme is known to be Mg-ATP, then the dependence on Mg^{++} ions may be affected not only by a modification of the regulatory receptors on which

5 A. A. White and T. V. Zenzer, Analyt. Biochem. *41*, 372 (1971).
6 T. Russel and I. Pastan, J. biol. Chem. *248*, 5835 (1973).
7 J. Otten, G. S. Johnson and I. Pastan, J. biol. Chem. *247*, 7082 (1972).
8 M. M. Burger, B. M. Bombik, B. M. Breckenridge and J. R. Sheppard, Nature New Biol. *239*, 161 (1972).
9 W. L. Ryan, N. A. Short and G. L. Curtis, Proc. Soc. exp. Biol. Med. *150*, 699 (1975).
10 H. J. Phillips and J. E. Terryberry, Exp. Cell Res. *13*, 341 (1957).
11 H. J. Phillips, Can. J. Biochem. *45*, 1495 (1967).
12 R. E. Scott, L. T. Furch and J. H. Kersey, Proc. nat. Acad. Sci. USA *70*, 3631 (1973).
13 S. Venuta and H. Rubin, Proc. nat. Acad. Sci., USA *70*, 653 (1973).
14 W. B. Anderson, G. S. Johnson and I. Pastan, Proc. nat. Acad. Sci. USA *70*, 1055 (1973).
15 W. B. Anderson, E. Lovelace and I. Pastan, Biochem. biophys. Res. Commun. *52*, 1293 (1973).
16 M. Yoshida, M. Owada and K. Toyoshima, Virology *63*, 68 (1975).

Mg^{++} ions act, but also by a modification of the catalytic receptor. Consequently, although trypsin acts on the dependence on Mg^{++} ions, we may not conclude exclusively that it changes the regulatory receptors. It may be that trypsin reacts directly with the catalytic receptors of adenylate cyclase, but we would rather suggest that limited proteolysis exposes receptors which were previously hidden. This activation lasts as long as cells restore their initial membranal organization. The membrane structure and functions of transformed cells are different from those of normal cells. Ultrastructural freeze fracture morphology[12] and activity, or certain membrane enzymes are changed[13]. Adenylate cyclase activity of transformed cells is lower than that of normal cells[14]. It has been suggested that the characteristics of the enzyme altered with different transformation agents[15,16]; consequently we cannot state, from our findings relating to the KB cells, that the mechanism of the action of trypsin on adenylate cyclase is the general rule. However, trypsination of cell cultures can affect cell metabolism by modifying the enzymatic system of adenylate cyclase; the use of another method of removing cells would appear preferable in order to eliminate some biochemical changes during studies of cell culture.

The generation and identification of the hemolysin of Trypanosoma congolense[1]

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Summary. The hemolytic activity of Trypanosoma congolense appears to be due to the presence of free fatty acids generated by the action of phospholipase A on endogenous phosphatidyl choline. Some lysolecithin also contributes to the lytic activity. Trypanosoma lewisi, being devoid of phospholipase A, does not generate free fatty acids and is therefore non-hemolytic.

Although the diseases caused by the African trypanosomes are of major economic and social significance, their pathogeneses remain very poorly understood[2]. Various hypotheses have been put foreword to account for the death of trypanosome infected animals including the suggestion that these organisms produce toxins[3-5]. Until recently, this was considered to be unlikely since the unequivocal occurrence of such toxins had not been demonstrated. It has now been shown however, that both Trypanosoma congolense[6] and T. brucei[7] generate, on autolysis, material which is potently cytotoxic and hemolytic.

Material and methods. Hemolytic activity is absent from freshly isolated T. congolense strain TREU 112, but is generated on incubation of a 7% v/v suspension of these organisms for 8–10 h at 20 °C in phosphate buffered glucose (PBG) (0.04 M, pH 8). At the end of this time, such a suspension is capable of causing 100% lysis of an equal volume of 2.5% sheep erythrocytes in PBG within 2 min at 37 °C. All hemolytic activity is contained within the particulate fraction of the suspension, being sedimented by centrifugation at 5,000 × g for 5 min. This autolysate is also capable of lysing rabbit buffy coat cells and mouse peritoneal cells as well as being able to cause a local acute inflammatory response on intradermal inoculation into rabbits[6].

In order to determine the nature of the hemolytic material, 1 ml of an autolysed trypanosome suspension prepared as described above and possessing hemolytic activity was extracted by shaking with chloroform-methanol (2:1) for 20 min at room temperature. At the end of this time the chloroform layer was separated, evaporated to dryness under vacuum at room temperature and resuspended to 1 ml in PBG. The methanol was removed from the aqueous layer by evaporation to its original volume. Hemolytic activity, as shown by 100% lysis of an equal volume of 2.5% sheep erythrocytes within 30 min, was found to be confined to the material extracted by chloroform. No lysis was observed in the aqueous fraction even after incubation with erythrocytes for 18 h at 37 °C.

A chloroform extract of autolysed trypanosomes was further analysed by thin layer chromatography. The material was streaked onto plates coated with silica gel H (Merck) of 0.5 mm thickness with a number of phospholipid and fatty acid standards. The chromatogram was developed in chloroform-methanol-water (65:25:4) and visualized in iodine vapour. The spots were identified against the standards, scraped off, and the scrapings extracted with two washings of chloroform-methanol-N HCl (20:10:1). The extracts were evaporated to dryness under vacuum and 0.2 ml 2.5% sheep erythrocytes in PBG added to each dried tube. Complete lysis of these erythrocytes occurred after 2 h at 37 °C in the presence of material identified as free fatty acid. Slight hemolysis (12%) was also observed in the presence of material identified as lysophosphatidylcholine.

Results and discussion. Preliminary results from gas chromatographic analysis of this free fatty acid fraction indicated that it consisted largely of stearic, palmitic, oleic and linoleic acids.

Several other features of the hemolytic process tend to confirm that lysis is primarily due to the activities of fatty acids and, to a lesser extent, lysolecithins. Thus trypanosome induced hemolysis is inhibited in the presence of fatty acid free bovine serum albumin[5]. It is well recognized that albumin has a strong affinity for free fatty acids[8] and lysolecithin[9], so that these compounds are no longer available to interact with cell membranes.

1 This research was supported by the International Development Research Center.

2 L. G. Goodwin, in: Trypanosomiosis and Leishmaniasis. Ciba found. symp. No. 20 (New series). Elsevier, Amsterdam 1973.
3 K. Landsteiner and H. Raubitscheck, Zentlb. Bakt., Orig. *45*, 660 (1907).
4 A. Laveran and A. Pettit, Bull. Soc. Path. exot. *4*, 42 (1911).
5 R. N. T. W. Fiennes, Ann. trop. Med. Parasit. *44*, 222 (1950).
6 I. R. Tizard and W. L. Holmes, Experientia, *32*, 1533 (1976).
7 C. N. Huan, Schweiz. med. Wschc. *105*, 1582 (1975).
8 W. Flehn and W. Hasselbach, Eur. J. Biochem. *13*, 510 (1971).
9 K. S. Cho and P. Proulx, Biochem. biophys. Acta *225*, 214 (1971).