ISOLATION AND IDENTIFICATION OF AGGREGATION PROMOTING SUBSTANCES FROM THE BOVINE EYE LENS*

M. IWIG, E. WEBER, E. FRIEDRICH
and D. GLAESSER
Institute of Physiological Chemistry and Department of Dermatology, Martin-Luther University, Halle (Saale), GDR

Received 1 December 1972
Original illustrations received 24 January 1973

1. Introduction

Cell aggregation is an essential process for cell differentiation in multicellular organisms [1]. The regulation of this process by physiological effectors is an interesting unsolved problem. In the cellular slime mould, *Dyctiostelium discoideum*, the aggregation and differentiation is regulated by the purine derivatives cyclic AMP and 5′ AMP [2]. Moreover adenine and adenine-containing nucleotides activate a differentiation-inducing protein, which induces the multiplication and differentiation of macrophages and granulocytes [3]. In this connection our finding of a strong aggregation stimulating effect of crude lens concentrate as well as synthetic C_6-substituted purine derivatives seems to be of some importance [4]. Our test system is different from others since the cells are not trypsinized and remain in connection with their native substratum. The aggregation of the lens epithelium cells can be observed after parts of the lens capsule with the adhering epithelium cells are transferred to the culture medium at 37°. The aggregation promoting substances enhance the accumulation of the originally single layered epithelium cells into cord and heap-like aggregates during the first 4 to 10 hr of tissue culturing and/or retard the spreading of the aggregated cells into a monolayer-like cell sheet [4]. This paper deals with the isolation and the identification of the aggregation stimulating substances from bovine eye lenses.

2. Materials and methods

2.1. Since the *aggregation promoting activity* of the crude lens homogenate turned out to be heat stable low molecular weight substances, we prepared a deproteinized lens concentrate as follows: Bovine eye lenses were prepared from young cattle (1–1.5 yr old) 1–2 hr after sacrifice. After removal of the lens capsule the fiber material of the lens cortex was loosened in distilled water (10 ml water per lens) by continuous stirring at room temp. The lens nuclei were discarded. The fiber material was homogenized at room temp. The homogenate was deproteinized by lowering the pH to 6.0 with 1 N HCl, followed by heating in boiling water for 30 min. After centrifugation at 12,000 *g* for 5 min the supernatant was concentrated 100-fold in a rotatory vaporizer at 50°.

2.2. *Ion exchange chromatography* with DEAE-Sephadex A-25 (Pharmacia) was used for the separation of the low molecular weight substances. Having regard to the biological test system we used volatile elution media. Elution was with water followed by an HCl gradient, linearly rising up to 0.2 N HCl. The elution absorbance profile was continuously recorded at 254 nm. The eluted fractions were dried in a rotatory

* Dedicated to Prof. Dr. Dr. S.M. Rapoport on his 60th birthday.

North-Holland Publishing Company – Amsterdam

vaporizer and dissolved in distilled water; the final concentration was 50–100 times higher than the *in vivo* concentration.

2.3. *Peptides* (peaks 1–5, fig. 1) were hydrolyzed with 6 N HCl at 105°, for 24 hr.

2.4. *Peptides and amino acids* (peaks 1–5) were separated by paper chromatography in sec. butanol/85% formic acid/water (75:15:10) and stained with ninhydrin [5].

2.5. *Hydrolysis of the nucleotides* was performed according to Marshak et al. [6] with concentrated perchloric acid (70%; D_{20} 1.67). After hydrolysis the perchloric acid was neutralized with a 12 N KOH and the precipitated potassium perchlorate discarded. The supernatant was dried and the nucleo bases were redissolved in 0.1 N HCl.

2.6. *Thin layer chromatography:* The layers were prepared by the methods of Mangold [7] and Randerath [8] with some minor modifications: 15 g Cellulose MN 300 G or MN 300 UV_{254} (Macherey, Nagel and Co.) were suspended in 40 ml ethanol (96%; v/v) and 50 ml distilled water; 10 g DEAE-Cellulose (Serva) in 60 ml distilled water and 8 g PEI-Cellulose (Serva) in 60 ml distilled water. If not otherwise noted 0.5 g of the fluorochromophore Leuchtpigment ZS-Super (Riedel DeHaen) were added. The chromatographic separations were performed on Cellulose MN 300 G with solvent systems A, B, C, D, on Cellulose MN 300 UV_{254} with E, on Cellulose MN 300 G (without Leuchtpigment) with F, G, on DEAE-Cellulose with H and on PEI-Cellulose with I, K, L. Solvent systems: (A) n-butanol/acetone/glacial acetic acid/ammonia/water (35:25:15:15:10; by vol.) [8]; (B) saturated ammonium sulfate/1 M sodium acetate/isopropanol (80:18:2; by vol.) [8]; (C) 0.1 M phosphate buffer (pH 6.8)/saturated ammonium sulfate/n-propanol (75:45:1.5; by vol.) [9]; (D) n-butanol/water (86:14; by vol.) [10]; (E) n-butanol/methanol/water/conc. ammonia (60:60:20:1; by vol.) [10]; (F) methanol/conc. HCl/water (70:20:10; by vol.) [10]; (G) isopropanol/conc. HCl/water (85:20.5:19.5; by vol.) [9]; (H) 0.02 N HCl [8]; (I) 0.25 M LiCl [10]; (K) 1 N CH_3COOH [10]; (L) 2% (v/v) H_3BO_3/2 M LiCl (2:1; v/v) [11].

After development the compounds were located on the chromatograms by short-wave ultraviolet light and marked on the plates. For reference the following compounds were used: nucleo bases: A†, G, X, C, U, T, (Serva); H (Reanal); ribonucleosides: Ado, Guo, Ino, Cyd, Urd (Serva); deoxyribonucleosides: dAdo, dGuo, dCyd, dUrd, dThd (Serva); ribonucleotides: Ado-5′-P, Ado-3′:5′-P, Guo-2′(3′)-P, Ino-5′-P, Cyd-5′-P, Urd-2′(3′)-P, Urd-5′-P (Serva) Ado-2′(3′)-P, Ado-5′-P_2, Ado-5′-P_3, Cyd-2′(3′)-P ((Reanal) deoxyribonucleotides: dAdo-5′-P, dGuo-5′-P, dCyd-5′-P, dUrd-5′-P, dThd-5′-P (Calbiochem); other compounds: NAD, NADH, NADP (AWD, Dresden), UDPG (Serva).

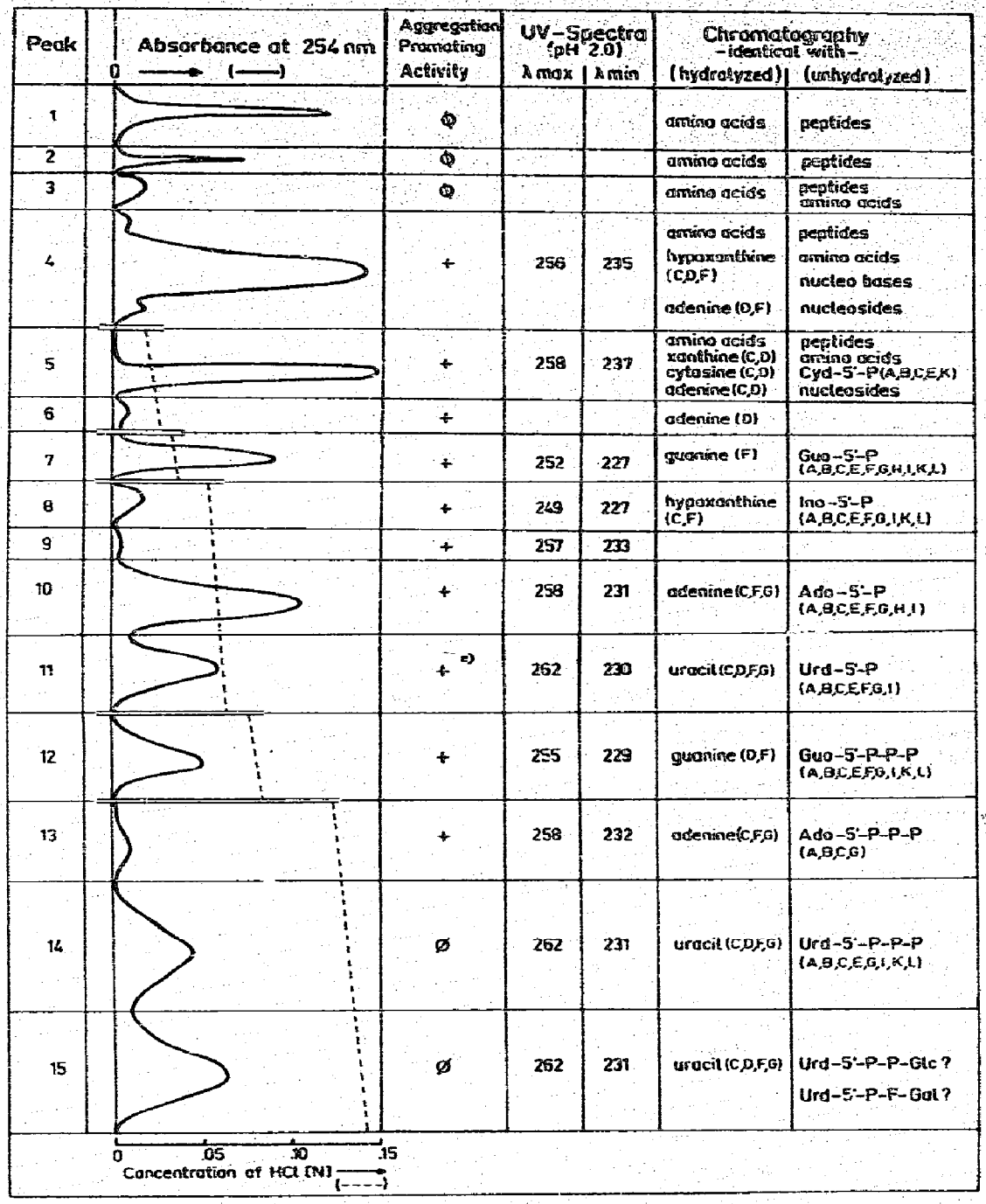
2.7. *Ultraviolet spectra* of the isolated substances were recorded with a continuously registrating spectrophotometer Unicam (SP 800) at pH 2.0.

2.8. *Testing of the isolated substances for aggregation promoting activity* was done as previously described [4].

3. Results

The elution pattern of the ultraviolet absorbing substances eluted from the DEAE-Sephadex column is given in fig. 1. This figure also summarizes the data obtained by UV-spectrophotometry, thin-layer chromatography, and the cell aggregation test. The crude lens concentrate was separated into 15 peaks. In the first line we have tried to identify the substances in those peaks which could be shown to have an aggregation promoting activity. The mixture of all the non-UV-absorbing material (the marked interspaces, fig. 1) was completely inactive regarding cell aggregation. Ten of the 15 peaks were active in stimulating aggregation. Five of them (7, 8, 10, 12, 13) contained a C_6-substituted purine nucleotide. The spectra of these substances were in good agreement with the results obtained by thin-layer chromatography. In agreement with the aggregation promoting activity of the corresponding synthetic compounds peak 7 (Guo-5′-P) stimulated the aggregation down to about 5×10^{-5} M.

† Abbreviations according to IUPAC-IUB tentative rules (1965).



Peak	Aggregation Promoting Activity	UV-Spectra (pH 2.0) λ max	λ min	Chromatography –identical with– (hydrolyzed)	(unhydrolyzed)
1	Φ			amino acids	peptides
2	Φ			amino acids	peptides
3	Φ			amino acids	peptides amino acids
4	+	256	235	amino acids hypoxanthine (C,D,F) adenine (D,F)	peptides amino acids nucleo bases nucleosides
5	+	258	237	amino acids xanthine (C,D) cytosine (C,D) adenine (C,D)	peptides amino acids Cyd-5'-P (A,B,C,E,K) nucleosides
6	+			adenine (D)	
7	+	252	227	guanine (F)	Guo-5'-P (A,B,C,E,F,G,H,I,K,L)
8	+	249	227	hypoxanthine (C,F)	Ino-5'-P (A,B,C,E,F,G,I,K,L)
9	+	257	233		
10	+	258	231	adenine (C,F,G)	Ado-5'-P (A,B,C,E,F,G,H,I)
11	+	262	230	uracil (C,D,F,G)	Urd-5'-P (A,B,C,E,F,G,I)
12	+	255	229	guanine (D,F)	Guo-5'-P-P-P (A,B,C,E,F,G,I,K,L)
13	+	258	232	adenine (C,F,G)	Ado-5'-P-P-P (A,B,C,G)
14	Ø	262	231	uracil (C,D,F,G)	Urd-5'-P-P-P (A,B,C,E,G,I,K,L)
15	Ø	262	231	uracil (C,D,F,G)	Urd-5'-P-P-Glc ? Urd-5'-P-F-Gal ?

Fig. 1. Elution pattern of the low molecular weight substances of the bovine eye lenses after chromatography on DEAE-Sephadex with data on aggregation promoting activity and analytical results. The solid line represents the absorbance at 254 nm and the broken line gives the concentration of HCl during chromatography. Capital letters in brackets give the solvent system by which the identity of the isolated substance with the synthetic compound was established. Besides the main component given there are impurities from peak 10 to which the aggregation promoting activity may be due.

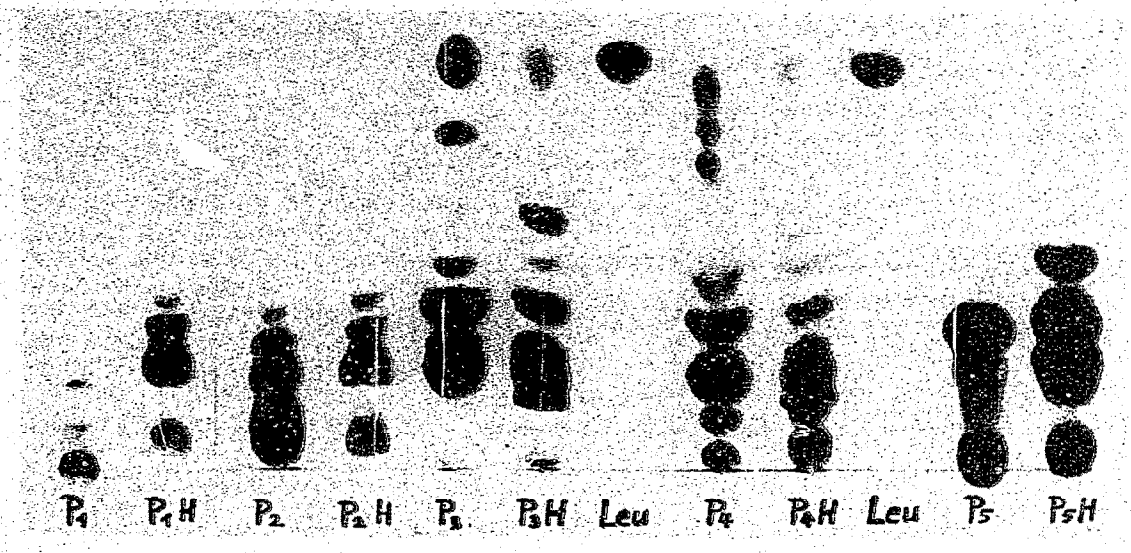


Fig. 2. Paper chromatographic separation of the ninhydrin-positive substances of peaks 1–5 before and after hydrolysis (H) with 6 N HCl; Leu = leucine.

peak 8 (Ino-5′-P) down to about 10^{-5} M and peak 10 (Ado-5′-P) down to about 5×10^{-6} M. Two active peaks (4 and 5) contained besides amino acids and peptides a mixture of different nucleobases and/or nucleosides. After hydrolysis the presence of C_6-substituted purines could be demonstrated. The material from peak 6 and 9 was not sufficient for a correct analysis. The aggregation stimulating effect of peak 11 (Urd-5′-P) is certainly due to impurities from peak 10 (Ado-5′-P) as synthetic uridine derivatives are without effect on the aggregation phenomenon [4]. Some results of peaks inactive regarding cell aggregation are also given in figs. 1 and 2.

4. Discussion

The aim of our investigations was the identification of the aggregation promoting substances from the lens. Therefore we did not attempt to completely identify all the isolated fractions. Our hope of finding any one substance with a high specificity regarding cell aggregation has not been fulfilled. All of the isolated aggregation promoting substances could be identified as naturally occurring nucleotides. But an important result is that the aggregation stimulating substances contained in the lens itself show the same specificity as it was found with synthetics [4]. All of the aggregation stimulating fractions contained C_6-substituted purine derivatives. Fractions containing amino acids, peptides or pyrimidine derivatives but no purines were completely ineffective. This finding points to the presence of specific receptor molecules. Moreover, the stimulation of cell aggregation with rather low concentrations of C_6-substituted purines, e.g. 2×10^{-6} M Ado-5′-P [4], demonstrates a high sensitivity for such effectors. There is much evidence that in our test system the C_6-substituted purines interfere with cell–substratum interactions [13]. The cell adhesion to the substratum is thought to play an important role for the regulation of cell movement [12]. A possible action of the effective purine derivatives on membrane bound enzymes or on enzyme activities of contractile proteins has been discussed [4, 13].

We are now looking for such enzymes sensitive to C_6-substituted purine derivatives.

Acknowledgements

We wish to thank Prof. H. Hanson for his generous support during the course of this work. The technical assistance of Miss G. Geiseler and Miss U. Mollenhauer is gratefully acknowledged.

References

[1] A. Moscona, in: Cells and Tissues in Culture, Vol. 1, ed. E.N. Willmer (Academic Press, London, New York, 1965) p. 489.
[2] H. Rasmussen, Science 170 (1970) 404.
[3] T. Landau and L. Sachs, FEBS Letters 17 (1971) 339.
[4] D. Glaesser, E. Friedrich, M. Iwig and E. Weber, Exp. Cell Res. 72 (1972) 409.
[5] S. Ansorge and P. Bohley, Pharmazie 22 (1967) 574.
[6] A. Marshak and H.J. Vogel, J. Biol. Chem. 189 (1951) 597.
[7] H.K. Mangold, in: Dünnschicht-Chromatographie, ed. E. Stahl (Springer, Berlin, New York, 1967) p. 749.
[8] K. Randerath, Dünnschicht-Chromatographie (Chemie, Weinheim, 1962) p. 183.
[9] J.E. Ciardi and E.P. Anderson, Analyt. Biochem. 22 (1968) 398.
[10] K. Randerath and E. Randerath, in: Methods in Enzymology, Vol. XII, A, eds. L. Grossman and K. Moldave (Academic Press, New York, 1967) p. 323.
[11] K. Randerath and E. Randerath, J. Chromat. 16 (1964) 111.
[12] J.P. Trinkaus, T. Betchaku, L.S. Krulikowski, Expti. Cell Res. 64 (1971) 291.
[13] D. Glaesser, M. Iwig, E. Weber and E. Friedrich, Studia Biophysica (1972) in press.