constant light were significantly ($p < 0.02$) lighter in body weight at day 7 than normal pups. No significant differences in body weight remained at day 21.

The percentage of parenchymal cells labeled with ^{3}H-thymidine 6 h after injection was 6.61% at day 1, 1.16% at day 7, 0.52% at day 14 and 0.10% at day 21 for normal animals. Data from the dark and light groups were not significantly different. Results from animals killed 6 to 14 days after injection substantiated the fact that mitotic activity continued, but decreased with age.

The percentage of parenchymal cells in the pineal gland decreased significantly ($p < 0.001$) from 98.44% at day 1 to 96.62% at day 21 for normal pups. There were no significant differences between groups. The percentage of neuroglial (normal, $p < 0.01$; dark, $p < 0.02$; light, $p < 0.05$) and endothelial (normal, $p < 0.01$; dark, $p < 0.001$; light, $p < 0.01$) cells increased significantly with time, but a significant increase in ependymal cells occurred only in the dark group ($p < 0.001$).

Parenchymal cell area increased in all groups, but some differences in timing occurred. For normal animals, a sudden and significant ($p < 0.01$) increase occurred between day 7 and 14. For rats in the dark group, significant increases in size occurred between day 1 and 7 ($p < 0.02$) and between day 14 and 21 ($p < 0.05$). For rats kept in constant light, no change in size of parenchymal cells occurred from day 1 to 7, but significant increase occurred between day 7 and 14 ($p < 0.02$) and day 14 and 21 ($p < 0.01$). On day 7 parenchymal cells of the dark group were significantly larger than those of the light group ($p < 0.01$) and at day 14 the parenchymal cells of the light group were significantly smaller than those of the normal group ($p < 0.05$).

Discussion. KERENYI and VON WESTARP[8] showed that constant darkness delayed the transformation of the pineal gland of rabbits, based on morphology. Our results using rats indicate that neither long or short photoperiods could significantly alter the postnatal mitotic activity of pineal parenchymal cells or the percentages of parenchymal, neuroglial, endothelial and ependymal cells from normal. The parenchymal cell area from rats in the dark group were increased at day 7 and for rats in the light group were decreased at day 14, but no significant differences were found on day 21. We conclude that length of photoperiod has little direct effect on the morphological maturation of the rat pineal gland in the early postnatal period, since the differences seen could be the result of alterations in the body weight gain.

However, the significant differences in the body weights of the pups in the different groups seems to indicate that photoperiod has a rapid and marked effect on lactation of the mother rats in the immediate postpartum period and that this may be a time when the pineal best shows its control of a hormonal pathway.

The decrease in weight gain of pups in the dark group between day 14 and 21 may indicate that the dramatic inhibition of growth hormone production and release by the pituitary gland in blinded rats shown by SORRENTINO, REITER and SCHALCH[9] for young rats may start as early as day 14.

[8] N. A. KERENYI and C. VON WESTARP, Endocrinology *88*, 1077 (1971).

[9] S. SORRENTINO, R. J. REITER and D. S. SCHALCH, Neuroendocrinology *7*, 210 (1971).

Immunological and radioimmunological studies of membrane antigen(s) from human breast carcinomas and non-tumoral breast tissues. I.

A. Bartorelli[1,2] and R. Accinni

Istituto di Ricerche Cardiovascolari dell'Università di Milano, via F. Sforza 35, I–20122 Milano (Italy),
21 September 1976

Summary. The authors extracted and partially purified a pool of antigens from primary breast carcinomas. The antigens responded to anti-CEA antibody in a radioimmunoassay (R. I. A.) and were not detected in non-tumoral breast tissues used as controls. Antisera were obtained by immunizing rabbits.

Introduction. In over 1000 cases investigated[3,4] by our direct R.I.A. of plasma carcinoembryonic antigen (CEA)[4,5] in patients with malignant tumors differing in type from adenocarcinomas of the gastroenteric tract, only 18 out of 243 cases gave positive results. Of these, 10 were carcinomas of the breast with metastasis (M^+). No case of adenocarcinoma of the breast without metastasis (M_0) was positive. The antigen used was extracted from hepatic metastasis of adenocarcinomas of the colon[3,6].

The small percentage of M^+ breast carcinomas positive to our R.I.A. suggests that in this pathology the circulating antigens show little reaction with our antibody. Therefore, the result is positive only in the few cases in which the blood level of these antigens is high.

Although no case of M_0 breast carcinoma was positive to our R.I.A. for CEA, we postulate that primary tumors of this type might have antigenic determinants that could be recognized by our antibody, however low the affinity. It will thus be possible to use this anti-CEA antibody to monitor the extraction and initial purification of antigens that are tumor-associated with primary breast carcinoma.

Materials and methods. A pool of histologically different primary breast carcinomas obtained by biopsy was used (MK). No metastatic tissue was used, as opposed to other

1 The authors want to express their appreciation to Dr Sergio Orefice, Prof. Carlo Mor and Dr Luisa Amante for their collaboration.

2 II Cattedra di Radiologia dell'Università di Milano.

3 A. Bartorelli, C. Mor, S. Orefice, R. Ferrara, C. Biancardi and R. Accinni, XXIVth Colloquium 'Protides of the Biological Fluids', Brugge 1976.

4 A. Bartorelli, R. Accinni, A. Golferini, A. P. Mistretta, G. C. Tassi, A. De Barbieri, C. Mor, G. Leonetti, S. Orefice and F. Rocco, Boll. Ist. sieroter. milan. *52*, 333 (1973).

5 A. Bartorelli, R. Accinni, C. Biancardi and R. Ferrara, Abstr. XIth Int. Cancer Cong. Florence *1*, 349 (1974).

6 A. P. Mistretta, A. Bartorelli, A. Golferini, G. C. Tassi, A. De Barbieri and R. Accinni, Experientia *30*, 1209 (1974).

authors[7]. Dysplastic or benign tumoral breast tissue obtained by biopsy was used as control (M). No normal glandular tissue, histologically negative glandular tissue at the periphery of the breast carcinoma, or contralateral normal glandular tissue in case of breast carcinoma was used. These tissues (MK and M) were homogenized, extracted with 3 M KCl in 5×10^{-3} M sodium-phosphate buffer, pH 7.4, and then centrifuged (45,000 × g for 1 h). The supernates were dialyzed exhaustively against distilled water and then lyophilized. Samples of the resulting crude extracts of MK and M were incubated with ^{125}I-CEA and absorbed[6] anti-CEA (goat 23) in order to obtain standard curves[4]. The crude extracts (MK and M) were resuspended in PBS, pH 7.2 (0.04 M sodium-phosphate + 0.1 M NaCl) at a concentration of 30 mg/ml. 1.5 ml of each solution was purified by gel-filtration on a 1.5 × 70 cm Sephadex G-200 column and eluted with PBS, pH 7.2 (flow rate: 4 ml/cm²/h). The protein content of each fraction was checked by determining the amount of ammonia nitrogen. 50 µl of each fraction was assayed with ^{125}I-CEA-anti-CEA.

Standard inhibition curves were constructed from the most active fractions 14, 15 and 16 of MK and fractions 13, 14, 15 and 16 of M using ^{125}I-CEA and anti-CEA-antiserum. The same fractions were also assayed against anti-CEA with the Ouchterlony double diffusion technique. The following 4 pools were formed with which 4 groups of 5 rabbits each were immunized: FMK (fractions 14, 15 and 16 of MK), PMK (fractions 11–16, comprising the first peak), FM (fractions 14, 15 and 16 of M) and finally PM (fractions 11–16 representing the whole of the first peak). Every 15 days each animal received 1 ml (600 µg/ml) of antigen emulsified with Freunds' complete adjuvant in a proportion of 1:1. After 35 days the antisera (anti-PMK, anti-FMK, anti-PM, and anti-FM) were assayed with the Ouchterlony immunodiffusion test against all the antigens used for the immunization.

Results. A considerable proportion of the ^{125}I-CEA in the ^{125}I-CEA-anti-CEA complex was displaced by the crude MK extract (figure 1a) when adding increasing amounts of antigen. Only a small proportion was displaced by the crude M extract. The curve obtained by plotting displace-

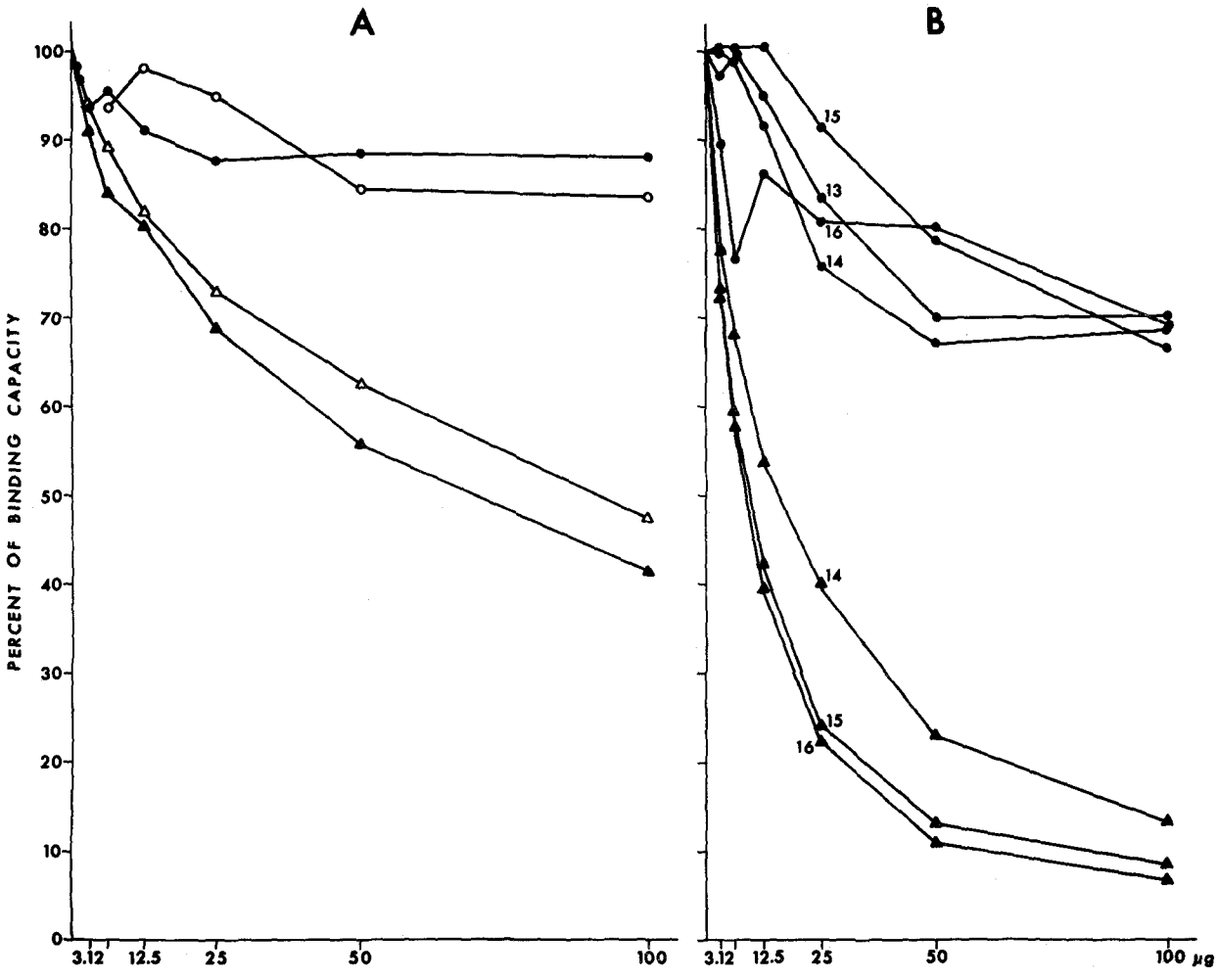

Fig. 1a. Standard inhibition curves obtained by incubating ^{125}I-CEA and anti-CEA with serial dilutions of crude MK and M extract. ●—● Crude extract from non-tumoral breast tissue (M). ▲—▲ Crude extract from primary breast carcinoma (MK). Material from a second extraction of M ○—○ and MK △—△.

Fig. 1b. Standard inhibition curves obtained by incubating ^{125}I-CEA and anti-CEA with serial dilutions of fractions 14, 15 and 16 of MK ▲—▲, and of fractions 13, 14, 15 and 16 of M ●—● after gel filtration on a Sephadex G-200 column.

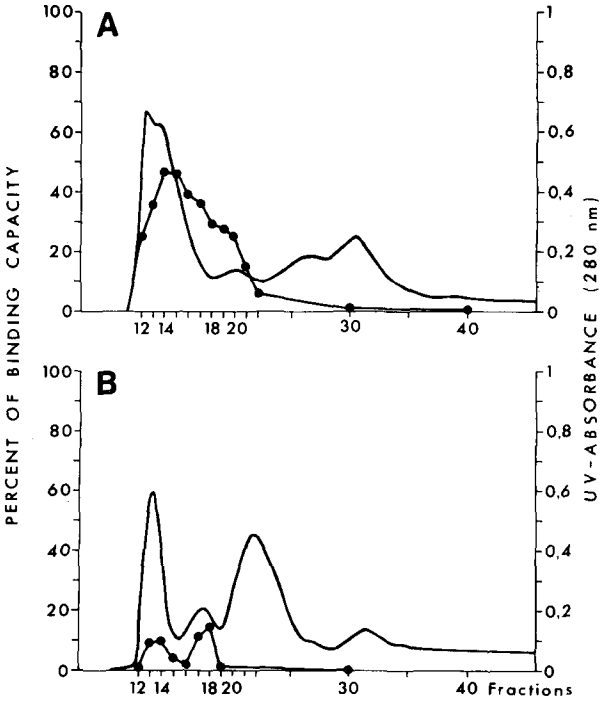

Fig. 2. — Profile of the elution of crude extracts of MK (A) and M (B) from a Sephadex G-200 column. ●—● Capacity of each fraction to bind anti-CEA.

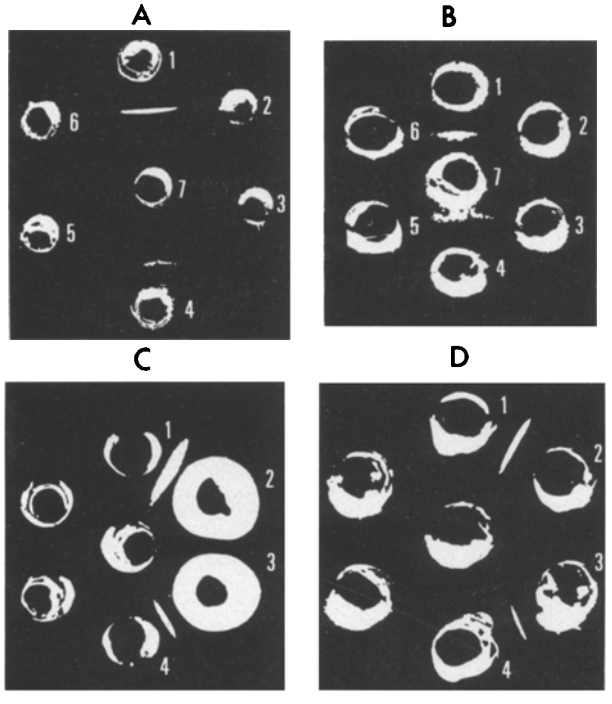

Fig. 3. Ouchterlony immunodiffusion pattern. *a* absorbed anti-CEA (goat 23); *b* unabsorbed anti-CEA (I P4). Both antisera (7) produced one major and one minor line when assayed against pure (1) or crude (4) CEA. No reactions were observed for the crude extracts MK (2) and M (3), and of MK purified by gel filtration PMK (5), FMK (6). *c* unabsorbed anti-PMK; *d* unabsorbed anti-FMK. Both antisera (2, 3) produced one major line when assayed against their respective antigens, PMK (C 1) and FMK (D 1), and one minor line when assayed against normal antigens PM (C 4) and FM (D 4).

ment as a function of increasing dilution was very flat and was unchanged by the addition of larger amounts of material (up to 1600 µg). Material from a second extraction gave the same results (figure 1a). Fractions 14 of MK and M, obtained by gel filtration of the crude extracts on a Sephadex G-200 column, had different optical densities at 280 nm (figures 2a and 2b).

In the R.I.A. with anti-CEA these fractions showed different binding capacities. The maximum binding capacity for fractions 14 and 15 of MK was 50% and did not correspond exactly to the peak (figures 2a and 2b). The standard inhibition curves show that the immune response to ^{125}I-CEA-anti-CEA was higher for fractions 14, 15 and 16 of MK (pooled = FMK) (figure 1b) than for the crude MK extract at equal protein concentrations (figure 1a).

In contrast, the response for fractions 13, 14, 15 and 16 of M (figure 1b) was similar to that of the crude M extract (figure 1a). The curves obtained for the fractions also show plateaus beginning with the first points. Fractions 14, 15 and 16 of MK (FMK), the first peak (PMK) obtained from gel filtration on Sephadex G-200 of MK and the crude extracts of MK and M produced no precipitation line when assayed by immunodiffusion against unabsorbed (I P4)[6] and absorbed (goat 23) anti-CEA antisera. A line was visible, however, obtained when these antisera were assayed against CEA (figures 3a and 3b). The precipitation line was strong for the reaction of anti-PMK and anti-FMK antisera with their antigens PMK and FMK (figures 3c and 3d) and weaker for the reaction with antigens from normal breast tissue (PM and FM) (figures 3c and 3d). No line was visible between the 2 antisera and CEA.

Discussion. The failure to demonstrate the presence of anti-CEA crossreacting antigen(s) in primary breast cancer extracts should probably be ascribed to the low sensitivity of the used methods. The anti-CEA crossreacting antigen(s) was, however, obtained from liver metastases of breast cancer. In this case the greater affinity of extracted antigens for anti-CEA would compensate for the low sensitivity of the methods[7]. In our opinion the reproducibility of the results of the extractions and immunological and radioimmunological assays indicate that primary breast carcinomas contain antigenic determinants for anti-CEA that seem to be absent from non-tumoral breast tissues. The immune response of MK after gel filtration was not due to interference phenomena; in fact the highest immunological activity was found in the descending portion of the first peak. Even when using unabsorbed anti-CEA, the Ouchterlony double diffusion test did not reveal antigens that were detectable by R.I.A. On the other hand, in the Ouchterlony test, CEA did not result in a precipitation line with unabsorbed anti-PMK and anti-FMK antisera. These antisera point to a distinction between their antigens and those of normal tissue.

7 T. Kuo, J. Rosai and T. W. Tillack, Int. J. Cancer *12*, 532 (1973).