6. M. Ben-David and A. Chrambach, *Endocrinology* **101**, 250 (1977).
7. P. Rathnam, L. Cederqvist, B. B. Saxena, *Biochim. Biophys. Acta* **492**, 186 (1977).
8. S. W. Walsh, R. K. Meyer, R. C. Wolf, H. G. Friesen, *Endocrinology* **100**, 845 (1977).
9. H. Friesen, P. Hwang, H. Guyda, G. Tolis, J. Tyson, R. Myers, in *Prolactin and Carcinogenesis*, A. R. Boyns and K. Griffiths, Eds. (Alpha Omega Alpha, Cardiff, 1972), p. 64.
10. D. H. Riddick and W. F. Kusmik, *Am. J. Obstet. Gynecol.* **127**, 187 (1977).
11. A. Golander, J. R. Barrett, L. Tyrey, W. H. Fletcher, S. Handwerger, *Endocrinology* **102**, 597 (1978).
12. Y. N. Sinha, F. W. Selby, U. J. Lewis, W. P. Vanderlaan, *J. Clin. Endocrinol. Metab.* **36**, 509 (1973).
13. J. I. Thorell and B. G. Johansson, *Biochim. Biophys. Acta* **251**, 363 (1971).
14. A. D. Rogol and S. W. Rosen, *J. Clin. Endocrinol. Metab.* **39**, 379 (1974).
15. R. P. C. Shiu *et al.*, *Science* **180**, 968 (1973).
16. S. Handwerger and L. M. Sherwood, in *Methods of Hormone Radioimmunoassay*, B. M. Jaffe and H. R. Behrman, Eds. (Academic Press, New York, 1974), p. 417.
17. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
18. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974).
19. Supported by NIH grant-HDO7447, the National Foundation-March of Dimes, PHS Research Career Development Award HD00069 (to S.H.), and PHS Special Research Fellowship HDO5508 (to T.H.). This work was presented in part at the 60th Annual Meeting of the Endocrine Society, Miami, Fla., June 1978.

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Tumor-Promoting Phorbol Esters Inhibit Binding of Epidermal Growth Factor to Cellular Receptors

Abstract. *Tumor-promoting phorbol esters and related plant macrocyclic diterpenes inhibit the binding of epidermal growth factor to its receptors on HeLa cells. This effect shows marked structural specificity and correlates with other biological effects of these compounds on mouse skin and in cell culture systems. The active compounds inhibited binding of ^{125}I-labeled epidermal growth factor with a 50 percent effective dose in the range of 10^{-8} to 10^{-9}*M. *Inhibition appears to be due to a decrease in the number of available epidermal growth factor receptors rather than a change in receptor affinity. These results suggest that certain biologic effects of tumor promoters may result from alterations in the function of cell surface receptors involved in growth regulation.*

Tumor-promoting agents are an intriguing class of compounds since, although they do not themselves induce cancer, they markedly enhance the production of skin tumors when repeatedly applied to mouse skin previously exposed to a low dose of a chemical carcinogen (*1*, *2*). Elucidation of the molecular action of tumor promoters would aid in the design of rapid screening systems for the detection of such agents in the human environment and would facilitate an understanding of the enigmatic multistage carcinogenic process. The most potent tumor promoter is 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). It is the active principle of croton oil (*1*, *2*), which was originally used as a promoting agent in the two-stage mouse skin carcinogenesis system developed by Berenblum and co-workers [see (*2*)].

Recent studies on mouse skin and with cell cultures have revealed a number of novel biologic and biochemical effects of TPA and related compounds (*1*-*3*). In cell culture systems TPA induces several phenotypic changes which resemble those seen in cells transformed by viruses or chemical carcinogens (*3*). This mimicry of transformation includes altered cell morphology and increased saturation density, alterations in lipid metabolism and cell surface glycoproteins, enhanced membrane transport of 2-deoxyglucose and possibly other nutrients, induction of the enzymes plasminogen activator and ornithine decarboxylase, and induction of prostaglandin synthesis (*4*). TPA also enhances the transformation of cells in culture previously exposed to a chemical carcinogen (*5*).

Another category of effects of TPA in cell culture is inhibition of terminal differentiation. This has been seen in cell cultures of diverse species and types and extends to a variety of programs of differentiation (*6*). Studies on mouse skin and epidermal cultures also suggest that TPA interferes with epidermal differentiation (*1*, *2*, *7*).

These and other findings have led us to postulate that TPA acts by usurping the function of a cell receptor or receptors whose normal function is to mediate the action of a yet to be identified endogenous growth regulator or hormone (*3*). Consistent with this hypothesis are (i) the low concentration at which TPA acts in cell culture (approximately 10^{-8} to $10^{-10}M$); (ii) the remarkable similarity in structural requirements seen when a variety of active and inactive phorbol esters and related macrocyclic diterpenes are tested in diverse systems; and (iii) the highly pleiotropic and reversible effects of these compounds. Since the earliest effects of TPA appear to occur at the cell membrane (*4*, *8*), we further postulated that the putative receptors are on the cell surface and the growth regulator may be a polypeptide hormone.

A possible candidate for the polypeptide hormone is epidermal growth factor (EGF), since it shares a number of biologic effects with TPA. These include stimulation of proliferation of both epidermal and mesodermal cells, increase in deoxyglucose transport (*9*), and induction of ornithine decarboxylase (*9*) and prostaglandin synthesis (*10*). In addition, EGF has been reported to promote tumor induction on mouse skin (*11*). We have recently found that like TPA, it is also a potent inducer of plasminogen activator production (*12*). EGF has been purified from both mouse and human sources. Mouse EGF has been extensively characterized. It contains 53 amino acids of known sequence (*13*). Like several other polypeptide hormones, EGF exerts its effects by binding to specific cell membrane receptors (*9*, *13*). Mouse and human EGF are not species-specific and compete with each other in receptor-binding studies, although they are antigenically different (*14*). The physiological role of EGF is not fully understood.

In the present study we have found that TPA and related macrocyclic diterpenes of plant origin are extremely potent inhibitors of EGF binding to intact mammalian cells.

The effects of various test compounds on the binding of ^{125}I-labeled EGF to HeLa cells are given in Fig. 1 and Table 1. Binding was almost completely inhibited by low concentrations (10 to 20 ng/ml) of unlabeled EGF, indicating that we were dealing with a specific saturable receptor. We found that extremely low concentrations (0.25 to 50 ng/ml) of TPA and related compounds produced a dose-dependent decrease in [^{125}I]EGF binding

Table 1. Effects of tumor promoters on cellular binding of [^{125}I]EGF. Binding assays were done with HeLa cell cultures essentially as described in Fig. 1. Test compounds were added at a concentration of 100 ng/ml to the binding buffer containing 0.225 ng of [^{125}I]EGF.

Test compounds	[^{125}I]EGF bound (count/min)
None	6149
12-*O*-tetradecanoyl phorbol-13-acetate (TPA)	255
Phorbol	5792
4-*O*-Me TPA	5510
Phorbol didecanoate	1349
Phorbol dibenzoate	2160
4αPDD	5956
Mezerein	620
Gnidipalmin	5457
Gnidimacrin 20-palmitate	5409
Gnilatimacrin	491
Gnidilatin	1042
Anthralin	6076

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(Fig. 1). At concentrations of 25 ng/ml ($4.2 \times 10^{-8}M$), TPA inhibited the binding of [^{125}I]EGF by about 90 percent. The relative potencies of a series of compounds and their median effective dose (ED_{50}) values were, in decreasing order: EGF, 1.2 ng/ml; TPA, 2.5 ng/ml; mezerein, 4.7 ng/ml; gnilatimacrin, 4.5 ng/ml; gnidilatin, 21.5 ng/ml; phorbol-12, 13-didecanoate (PDD), 30 ng/ml; and phorbol dibenzoate (PDB), 75 ng/ml. Phorbol, 4α-PDD, 4-*O*-methyl TPA (4-*O*-Me TPA), gnidipalmin, and anthralin did not have an appreciable effect on [^{125}I]EGF binding when tested at concentrations as high as 100 ng/ml (Table 1). The inhibitory effect, therefore, occurs at concentrations as low as 10^{-8} to $10^{-9}M$ and possesses marked structural specificity and stereospecificity for the test compounds. In addition, the structural requirements of the plant diterpenes for inhibition of [^{125}I]EGF binding parallel, in general, the requirements previously noted for induction of plasminogen activator (*4*, *15*) inhibition of differentiation of murine erythroleukemic cells (*6*), and other effects in vitro (*16*). On the basis of available data, our results also parallel the structural requirements for promoting activity in the two-stage mouse skin carcinogenesis assay (*17*). Thus, TPA is a potent promoter and PDD and PDB are less active promoters on mouse skin (*17*), whereas phorbol, 4-*O* Me TPA, and 4α-PDD are inactive. Anthralin is a promoter on mouse skin but only at concentrations about 10^3 times higher than the phorbol esters (*7*).

In all the above studies TPA or other test compounds were added to cells simultaneously with the [^{125}I]EGF. Inhibition of EGF binding was also observed if cells were exposed to TPA for 90 minutes, washed to remove unbound material, and then incubated with [^{125}I]EGF. This provides evidence that the inhibitory effect of TPA is on cellular components and cannot be attributed simply to the formation of an inactive TPA-EGF complex in the incubation medium. To further assess the mechanism of TPA inhibition of EGF binding to HeLa cells, we performed a Scatchard analysis (Fig. 2). These data were obtained after HeLa cell cultures were incubated for 50 minutes with increasing amounts of [^{125}I]EGF in the absence or presence of TPA (2.5 ng/ml). They represent equilibrium values, since we found that in HeLa cells EGF binding reaches an equilibrium at about 45 minutes after the addition of [^{125}I]EGF. It remains stable for an additional 40 minutes and after that time the amount bound declines. The analysis indicated that the number of EGF receptors per HeLa cell is 43,500, with a dissociation constant of $1.25 \times 10^{-10}M$. These values are in the range of those obtained with certain other cell types (*18*). In the presence of 2.5 ng of TPA per milliliter the apparent number of EGF receptors was reduced to 22,200 but the binding constant remained the same. Thus TPA leads to a decrease in the number of EGF receptors available for [^{125}I]EGF but does not alter the binding affinity of them. The data do not clearly distinguish whether this is a direct effect due to binding of TPA to EGF receptors or an indirect effect—for example, TPA-induced changes in cell membrane structure that might cause masking or degradation of EGF receptors so that they are unavailable for EGF binding. Consistent with an indirect mechanism is the evidence that TPA results in a number of changes in cell membrane structure and function (*4*).

To what extent can the various biologic effects of TPA be explained by its effects on the function of EGF receptors? The present results are not confined to HeLa cell cultures because in recent studies we have found that TPA also inhibits the binding of [^{125}I]EGF to human XPI fibroblasts (ATCC-CRL 1162) and mouse 3T3 cells (*19*) [unpublished studies].

Since EGF and TPA share many biologic effects, it is possible that some of the effects of TPA could result from a triggering of the EGF effector system.

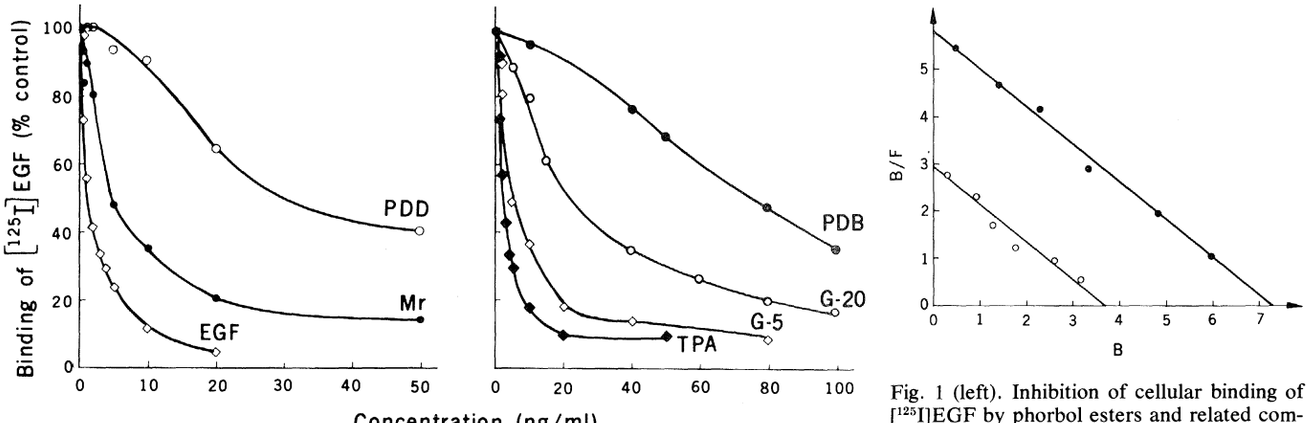

Fig. 1 (left). Inhibition of cellular binding of [^{125}I]EGF by phorbol esters and related compounds. The binding assays were performed on subconfluent cell culture monolayers in 5-cm petri dishes (Nunc) according to Todaro *et al.* (*20*). HeLa cells (~6×10^5 cells per dish) were seeded in minimum Eagle's medium (MEM) containing 10 percent calf serum 72 hours before the binding assay. The cells were then washed twice with 2.5-ml portions of MEM lacking serum and incubated with 0.17 ng of [^{125}I]EGF (mouse, specific activity 80 μCi/μg, Collaborative Research) in 1.5 ml of binding buffer per dish. Binding buffer consisted of Dulbecco's modified MEM (DMEM) containing bovine serum albumin (1 mg/ml) and 50 m*M* 2-[bis-(2-hydroxyethyl)amino]ethanesulfonic acid (Bes), *p*H 6.8. After incubation for 50 minutes at 36°C, the above medium was removed and the cells washed three times with 5-ml portions of cold DMEM. The cells were then solubilized with 1 percent Triton-X-100 and 1 percent sodium dodecyl sulfate and assayed for radioactivity in a liquid scintillation counter programmed for counting ^{125}I. Nonspecific binding was measured by the amount of cell-bound radioactivity in the presence of unlabeled EGF (1500 ng per plate). It represented about 1 to 2 percent of the total [^{125}I]EGF added, and this value was subtracted from all binding assay data. The structures and sources of the test compounds are described elsewhere (*15*). They were added to the binding buffer medium simultaneously with [^{125}I]EGF, at the final concentrations given on the abscissa. Cell binding of [^{125}I]EGF (in the absence of test compound) was about 5000 counts per minute per plate. Data are expressed as percentage of the control, that is, [^{125}I]EGF binding in the absence of the test compounds. Abbreviations: *Mr*, mezerein; *G-5*, gnidmacrin. Fig. 2 (right). Scatchard plot of [^{125}I]EGF binding and inhibition of binding by TPA. Binding assays were as described in Fig. 1 but were done with various concentrations (0.25 to 8.0 ng/ml) of [^{125}I]EGF. Saturation of the EGF receptor occurred with about 25 ng of [^{125}I]EGF per milliliter (4.2×10^9M). Bound [^{125}I]EGF (*B*) is expressed as (picomoles per 10^6 cells) $\times 10^2$. The concentrations of [^{125}I]EGF in the binding solution (*F*) is in molarity. All data are corrected for nonspecific binding (see legend for Fig. 1). Symbols: •, binding of [^{125}I]EGF; ○, binding of [^{125}I]EGF in the presence of 2.5 ng of TPA per milliliter.

The finding of Todaro *et al.* that murine cells transformed by sarcoma viruses have a decrease in EGF receptors (*20*) further suggests that changes in the EGF effector system may play an important role not only in the carcinogenic process but also in maintenance of the transformed state. On the other hand, we must emphasize that it is not clear that all of the actions of TPA are shared by EGF. In addition, there are cell types that appear to lack EGF receptors yet are responsive to TPA (*19, 21*). It seems likely, therefore, that the diverse biologic effects of TPA are not mediated entirely by the EGF receptor system. The ability of TPA to produce EGF-like effects on cell function may reflect a more generalized alteration in cell membrane structure and function induced by TPA. Nevertheless, even such secondary effects of TPA could lead to alterations in growth control.

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References and Notes

1. For comprehensive reviews on tumor promotion, see R. K. Boutwell, *CRC Crit. Rev. Toxicol.*, No. 419 (1974); E. Hecker, in *Handbuch der Allgemeinen Pathologie*, E. Grundmann, Ed. (Springer Verlag, Berlin, 1975), vol. 4, No. 6, p. 651; B. L. Van Duuren, *Prog. Exp. Tumor Res.* **11**, 31 (1969).
2. I. Berenblum, in *Cancer*, F. F. Becker, Ed. (Plenum, New York, 1975), vol. 1, p. 323.
3. I. B. Weinstein, M. Wigler, C. Pietropaolo, in *Origins of Human Cancer*, H. Hiatt, J. D. Watson, J. A. Winston, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977), vol. 4, p. 751; I. B. Weinstein, M. Wigler, P. B. Fisher, E. Sisskin, C. Pietropaolo, in *Carcinogenesis*, vol. 2, *Mechanisms of Tumor Promotion and Cacarcinogenesis*, T. J. Slaga, A. Sivak, R. K. Boutwell, Eds. (Raven, New York, 1978), pp. 313-333.
4. P. E. Driedger and P. M. Blumberg, *Cancer Res.* **37**, 3257 (1977); L. R. Rohrschneider and R. K. Boutwell, *ibid.* **23**, 1945 (1973); A. Sivak, F. Ray, B. L. Van Duuren, *ibid.* **29**, 624 (1969); M. Wigler and I. B. Weinstein, *Nature (London)* **259**, 232 (1976); S. H. Yuspa, U. Lichti, T. Ben, E. Patterson, H. Hennings, T. J. Slaga, N. Colburn, W. Kelsey, *ibid.* **262**, 402 (1976); L. Levine and A. Hassid, *Biochem. Biophys. Res. Commun.* **79**, 477 (1977).
5. S. Mondal, D. W. Brankow, C. Heidelberger, *Cancer Res.* **36**, 2254 (1976).
6. H. Yamasaki, E. Fibach, U. Nudel, I. B. Weinstein, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3451 (1977); R. Cohn, M. Pacifici, N. Rubinstein, J. Biehl, H. Holtzer, *Nature (London)* **266**, 538 (1977); G. Rovera, T. G. O'Brien, L. Diamond, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2894 (1977); L. Diamond, T. O'Brien, G. Rovera, *Nature (London)* **269**, 347 (1977).
7. A. N. Raick, *Cancer Res.* **33**, 269 (1973); N. Colburn, S. Lau, R. Head, *ibid.* **35**, 3154 (1975); S. H. Yuspa, T. Ben, E. Patterson, D. Michael, K. Elgjo, H. Hennings, *ibid.* **36**, 4062 (1976).
8. C. E. Wenner, J. Hackney, H. K. Kinelberg, E. Mayhew, *ibid.* **34**, 1731 (1974).
9. J. K. Hoober and S. Cohen, *Biochim. Biophys. Acta.* **138**, 347 (1967); S. Cohen, *Dev. Biol.* **12**, 394 (1965); D. Barnes and S. P. Colowick, *J. Cell. Physiol.* **89**, 633 (1976); M. Stastny and S. Cohen, *Biochim. Biophys. Acta.* **204**, 578 (1970).
10. L. Levine and A. Hassid, *Biochem. Biophys. Res. Commun.* **76**, 1181 (1977).
11. S. P. Rose, R. Stahn, D. S. Passovoy, H. Hershman, *Experientia* **32**, 913 (1976).
12. L. S. Lee and I. B. Weinstein, *Nature(London)* **274**, 696 (1978).
13. C. R. Savage, Jr., T. Inagami, S. Cohen, *J. Biol. Chem.* **247**, 7612 (1972); H. Gregory, *Nature (London)* **257**, 325 (1975); H. A. Armelin, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2702 (1973); M. D. Hollenberg and P. Cuatrecasas, *ibid.*, p. 2964.
14. G. Carpenter and S. Cohen, *J. Cell Biol.* **71**, 159 (1976); S. Cohen, G. Carpenter, K. J. Lembach, *Adv. Metab. Disord.* **8**, 265 (1975); S. Cohen and G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1317 (1975).
15. M. Wigler, D. DeFeo, I. B. Weinstein, *Cancer Res.* **38**, 1434 (1978).
16. L. Diamond, S. O'Brien, C. Donaldson, Y. Shimizu, *Int. J. Cancer* **13**, 721 (1974); R. G. Suss, G. Kreibich, V. Kinzel, *Eur. J. Cancer* **8**, 299 (1972); L. R. Rohrschneider and R. K. Boutwell, *Nature (London)* **243**, 212 (1973).
17. E. Hecker, *Cancer Res.* **28**, 2338 (1968); R. Langenbach and C. Kuszynski, *J. Natl. Cancer Inst.* **55**, 801 (1975); F. G. Bock and R. Burns, *ibid.* **30**, 393 (1963); W. M. Baird and R. K. Boutwell, *Cancer Res.* **31**, 1074 (1971).
18. B. Westermark, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1619 (1977).
19. L. S. Lee and I. B. Weinstein, in preparation.
20. G. J. Todaro, J. E. DeLarco, S. Cohen, *Nature (London)* **264**, 26 (1976).
21. R. W. Pruss and H. R. Herschman, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3918 (1977).
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The Attention Operating Characteristic: Examples from Visual Search

Abstract. *Even in the absence of eye movements, we show that subjects are able, upon instruction, to selectively attend to certain kinds of targets and parts of visual arrays. The major mechanism of altering attention is the switching of attention from trial to trial, although intermediate states of shared attention do occur. Attention operating characteristics are shown to be a useful way of describing such data and of assessing the compatibility of tasks to be performed simultaneously.*

The primary mechanisms of visual attention are overt physical acts: turning the eyes, head, and body toward the object of attention. Nevertheless, within a single eye fixation, attention can determine what parts of a complex stimulus will be remembered (*1*) and, in a simple reaction-time task, which reaction-stimuli will elicit fast and which will elicit slow responses (*2*). In contrast to these results in memory and reaction-time tasks (which involve complex short-term memory and response processes), in visual detection tasks (which primarily involve perceptual mechanisms) there is, as yet, no evidence that selective attention can alter processing within a brief exposure. In fact, there are many experiments that have been interpreted to mean that attention does not play an important role in detection (*3, 4*).

To demonstrate the crucial role of attention in visual detection, we used a variant of the classical visual search technique. In classical visual search, the subject examines an array of stimuli (background objects) for a target object by moving his eyes over the array (*5*). While the pattern of eye movements is interesting in itself (*6*), it adds a complication not under experimental control to the analysis of attention. Therefore, in our experiments eye movements are eliminated by having the subject keep his eyes fixated on the center of a display and presenting new stimuli to him every 240 msec. This method allows precise control over the flow of visual stimuli while approximating the sequence the eyes produce for themselves in spontaneous visual search.

We present a sequence of arrays of alphanumeric characters, preceded by a fixation field, on a cathode-ray tube (*7*). The subject's task is to detect two numerals (the target characters) among uppercase letters of the alphabet (background characters). The targets occur in only one array, the critical array. This is preceded by a random number (from 7 to 12) of noncritical arrays and followed by at least 12 more noncritical arrays. The subject does not know which array will contain the target characters, nor which of the ten numerals will occur, nor where in the array they will be located. His task is to report the identity and location of each of the target characters and his degree of confidence in the correctness of each report (*8*, p. 209).

In a previous study (*3*) using a similar paradigm, Sperling *et al.* observed that a subject can scan for an unknown one-often numeral as effectively as for a particular known numeral. They concluded that subjects scan for ten numerals in parallel. They further noted that subjects can scan 15 to 25 locations of an array in parallel. The purpose of the present experiment was to determine whether subjects could selectively attend to certain parts and kinds of targets in the array, even in a situation in which eye movements (should they occur) would be of no benefit.

We used the experimental paradigm described above, in which the critical array contained two different target numerals, chosen independently (*9*). The subject's task was to report both numerals, both locations, and two confidence ratings. Sample arrays are shown at the top

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