Other possible explanations of our observations have to be considered. In altering the deformability of erythrocytes[19], chlorpromazine might affect their flow properties such as to diminish the probability of platelet collisions. Any such effect is unlikely to make a measureable difference in our experiments; nor does it overcome the requirement that the platelets have to be activated before aggregating as an haemostatic plug. Another possibility is that chlorpromazine interferes with the aggregation of platelets after their activation by ADP, for example by competing with calcium[20] which is essential for aggregation. If this were the explanation, however, primary aggregation by added ADP should be inhibited by chlorpromazine in platelet-rich plasma, and this is not so[12]. Furthermore, the concentrations of chlorpromazine which prolong the bleeding time are much lower than the calcium concentration required for platelet aggregation, even in the presence of citrate as anticoagulant.

Direct flow-mechanical activation of platelets has been demonstrated experimentally but in very different conditions[21]. Haemodynamic disturbances produce platelet thrombi in the blood channels of artificial organs such as kidneys or oxygenators, whereby their function is often terminated prematurely[22]. The conclusion that the red cells are primarily responsible for these thrombi accords with the fact that thrombus formation is similar in channels made of various materials as long as the geometry is the same. Our results, therefore, suggest a new way of prolonging the function of such artificial organs through the addition of an agent capable of inhibiting the release of ADP (and ATP) from erythrocytes. Such an agent should, of course, have as few other effects as possible, so that chlorpromazine, although effective, would not be the choice.

Our observations similarly suggest a new approach to the prophylaxis of those thrombotic diseases in which thrombus formation depends on platelets, as in a proportion of acute coronary thromboses. This approach would require the demonstration that the incidence of such a disease is diminished by drugs which, in clinically acceptable blood concentrations, do not inhibit platelet function directly and which inhibit the release of ADP experimentally from non-haemolysing erythrocytes during rheological stress. Our hypothesis may also explain the effect of dipyridamole and sulphinpyrazone in preventing increased utilisation of circulating platelets in potentially thrombogenic conditions[23]. This cannot easily be accounted for by any direct action on platelets by either drug at its clinically effective concentration; instead, these drugs may act on the red cells to diminish their activation of platelets.

We thank Dr W. A. M. Duncan of Smith, Kline and French Ltd for SKF 525A.

G. V. R. Born

Department of Pharmacology, University of Cambridge, Cambridge CB2 2QD, UK

D. Bergquist
K.-E. Arfors

Department of Experimental Medicine, Pharmacia AB, Uppsala, Sweden

Received September 26; accepted December 3, 1975.

1 Gaarder, A., Jonsen, J., Laland, S., Hellem, A., and Owren, P. A., *Nature*, **192**, 531–532 (1961).
2 Haut, M. J., Cowan, D. H., and Harris, J. W., *J. lab. clin. Med.*, **82**, 44–53 (1973).
3 Peytremann, R., Rhodes, R. S., and Hartmann, R. C., *Ser. Haemat.*, **5** (3), 115–136 (1972).
4 Jorgensen, S., *Acta Pharm. Tox. Kbh.*, **12**, 294–302 (1956).
5 Ireland, D. M., and Mills, D. C. B., *Biochem. J.*, **99**, 283–296 (1966).
6 Haslam, R. J., and Mills, D. C. B., *Biochem. J.*, **103**, 773–784 (1967).
7 Fredrich-Freksa, H., Simon, A., and Pachowsky, H., *Naturforsch.*, **28c**, 195–197 (1973).
8 Schmid-Schönbein, H., Wells, R. E., and Goldstone, J., *Circulation Res.*, **25**, 131–143 (1969).
9 Harrison, M. J. G., and Mitchell, J. R. A., *Lancet*, **ii**, 1163–1164 (1966).
10 Downie, H. G., Murphy, E. A., Rowsell, H. C., and Mustard, J. F., *Circulation Res.*, **12**, 441–448 (1963).
11 Seeman, P., *Pharmac. Rev.*, **24**, 583–655 (1973).
12 Mills, D. C. B., and Roberts, G. C. K., *Nature* **213**, 35–38 (1967).
13 Lee I. P., Yamamura, H. I., and Dixon, R. L., *Biochem. Pharmac.*, **17**, 1671–1681 (1968).
14 Turitto, V. T., and Baumgartner, H. R., *Microvasc. Res.*, **9**, 335–344 (1975).
15 Begent, N. A., and Born, G. V. R., *Nature*, **227**, 926–930 (1970).
16 Richardson, P. D., *Nature*, **245**, 103–104 (1973).
17 Arfors, K.-E., Cockburn, J., and Gross, J., *Microvasc. Res.* (in the press).
18 Born, G. V. R., and Cross, M. J., *J. Physiol., Lond.*, **168**, 178–195 (1963).
19 Braasch, D., and Rogausch, H., *Pflügers Arch. ges. Physiol.*, **323**, 41–49 (1971).
20 Kwant, W. O., and Seeman, P., *Biochim. biophys. Acta*, **193**, 338–349 (1969).
21 Brown, C. H., Leverett, L. B., Lewis, C. W., Alfrey, C. P., and Hellums, J. D., *J. lab. clin. Med.*, **86**, 462–471 (1975).
22 Richardson, P. D., *Bull. N.Y. Acad. Med.*, **48**, 379–382 (1972).
23 Packham, M. A., and Mustard, J. F., in *Platelets; Production, Function, Transfusion and Storage* (Gruner and Stratton, New York, 1974).

Inhibition of plasmin-mediated fibrinolysis by vitamin E

Biological effects attributed to vitamin E include the maintenance of cell membrane integrity, inhibition of enzyme-dependent lipid peroxidation, participation in oxidative phosphorylation, and a general, non-enzymatic antioxidant effect[1]. In spite of these observations, the physiological function of the vitamin remains obscure. Natural deficiency in man has been invoked in the pathogenesis of several disorders, with convincing evidence limited to a specific type of haemolytic anaemia[2]. Therapeutically, vitamin E has its advocates in treatment of peripheral vascular and thromboembolic disease, although documentation for its efficacy is meagre[3]. Although it is considered non-toxic[4], little is known about adverse effects of excessive dietary vitamin E.

Plasminogen is the plasma proenzyme which, on conversion to its active form, plasmin, is considered responsible for lysis of fibrin deposits resulting from physiological or pathological activation of the coagulation cascade[5]. In spite of interest in vitamin E as therapy for vascular disease, and the importance of coagulation and fibrinolysis in intravascular homoeostasis[5], information on its effects on these processes is limited. An anti-thrombin effect has been claimed[6], but not confirmed[3]. Prolongation of plasma clotting time has been reported with vitamin E therapy, together with decrease in the expected increment in blood fibrinolytic activity which follows venous occlusion[7]. We report a direct effect of vitamin E on the fibrinolytic system, namely, *in vitro* inhibition of plasmin-mediated fibrinolysis at physiological concentrations of the vitamin and enzyme.

Fibrinolysis was assayed by a sensitive solid phase ^{125}I-fibrin tube method. This technique, described in detail elsewhere[8], is based on release of radioactive degradation products from ^{125}I-fibrin (human), originally absorbed to polystyrene tubes as fibrinogen, and converted to non-cross linked fibrin by thrombin. Plasminogen was isolated from normal human plasma by affinity chromatography on lysine–Sepharose[9], and subsequent stepwise[9] or gradient elution[10] with ε-aminocaproic acid (EACA). Plasmin was

Table 1 Effect of vitamin E on lysis of ^{125}I-fibrin by plasmin and other proteolytic enzymes

	^{125}I-fibrin lysed (c.p.m.)		
	− Vitamin E	+Vitamin E	Δ
Plasmin	9,968	1,634	−84%
Collagenase (Clostridiopeptidase A, Worthington)	18,941	17,870	− 6%
Chymotrypsin (α-chymotrypsin, bovine pancreas, Worthington)	36,070	39,539	+10%
Trypsin (bovine pancreas, Worthington)	38,380	44,363	+16%
Pronase (bacterial protease, type VI, Sigma)	51,001	67,864	+33%

Enzyme (5 μg ml^{-1}) and vitamin E (1.7×10^{-5} M) were incubated in ^{125}I-fibrin coated assay tubes for 30 min at 37 °C (final volume, 0.2 ml in Tris–NaCl buffer). Results are means of duplicate assays. Assay tubes contained 145,000 c.p.m. of ^{125}I-fibrin (90,000 c.p.m. per μg fibrin).

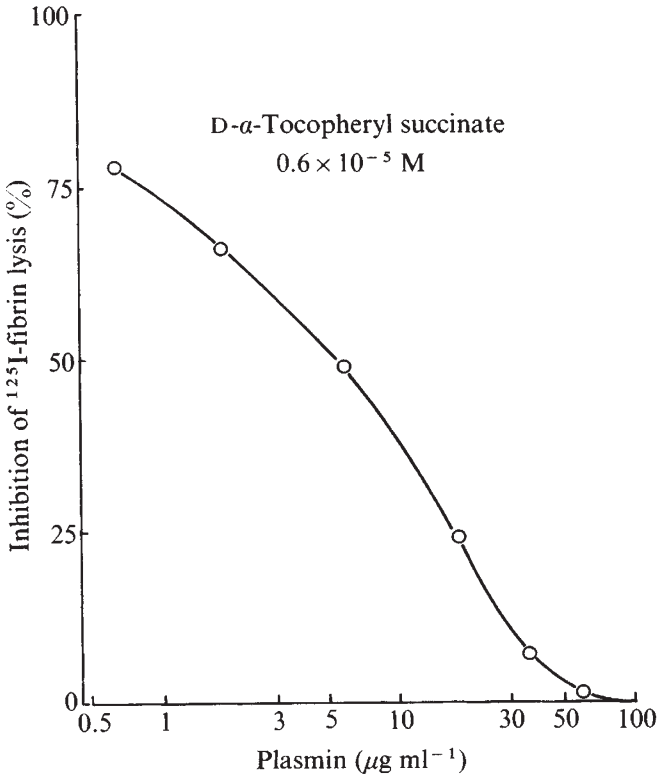


Fig. 1 Effect of vitamin E on lysis of ^{125}I-fibrin by human plasmin. Plasmin, at varying concentrations, was incubated with or without vitamin E (0.6×10^{-5} M) at 37 °C for 30 min (final volume, 0.2 ml) in assay tubes coated with ^{125}I-fibrin (140,000 c.p.m. per tube, 90,000 c.p.m. per μg fibrin). Lysis in presence of vitamin E is expressed as percentage of lysis by plasmin alone. Nonspecific release of radioactivity with buffer alone (289 c.p.m.) was subtracted from experimental values. Release with vitamin E alone (2.5×10^{-5} M), 270 c.p.m. Experimental values (means of duplicate assays) at representative plasmin concentrations (0.6, 6 and 60 μg ml^{-1}, values with vitamin E in parentheses): 364 (84), 6,504 (3,205) and 12,684 (11,870) c.p.m., respectively.

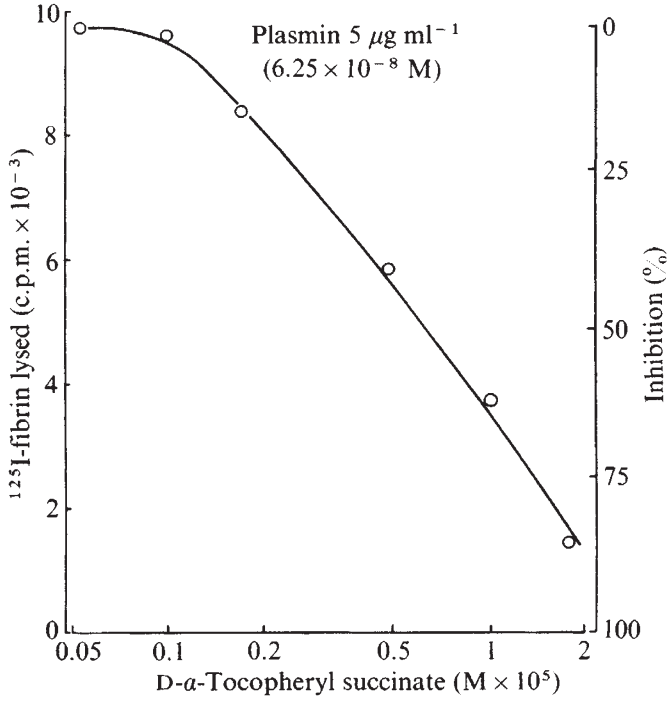
in the form of spontaneously activated plasminogen[8], as judged by absence of generation of additional activity by appropriate streptokinase or urokinase treatment. Plasminogen and plasmin were quantitated by absorbancy at 280 nm (ref. 11). Vitamin E, in the form of D-α-tocopheryl succinate (type VI, Sigma) was suspended in Tris–NaCl buffer (0.015 M Tris, 0.15 M NaCl, *p*H 7.4) at a concentration of 1 mg ml^{-1}, stirred for 15 min at room temperature, and filtered through Whatman No. 1 paper. Vitamin E content of the filtrate was quantitated by absorbancy at 292 nm (ref. 12), and colorimetrically by the tocopherol red reaction[13], after preliminary alkaline saponification to yield the free phenol. Concentrations of vitamin E obtained in this way were 3×10^{-5} to 4×10^{-5} M.

Vitamin E (0.6×10^{-5} M) produced 50% inhibition of fibrinolysis at a plasmin concentration of 5 μg ml^{-1}, or 0.6×10^{-7} M (molecular weight of plasmin, 81,000 (ref. 14), an inhibitor–enzyme molar ratio of 100 : 1 (Fig. 1). Examination of the effects of varying concentrations of vitamin E (plasmin, 5 μg ml^{-1}) was confirmatory (Fig. 2), with 50% inhibition at a vitamin E concentration of 0.5×10^{-5} to 0.6×10^{-5} M. Sodium succinate (5×10^{-5} M) or filtered buffer had no effect. Since vitamin E functions as an antioxidant[1], L-cysteine hydrochloride was tested. No effect on fibrinolysis was observed at concentrations from 10^{-7} to 10^{-3} M. Pretreatment of assay tubes with vitamin E (2×10^{-5} M, 37 °C, 30 min) had no effect on subsequent ^{125}I-fibrin lysis. The effect of vitamin E on fibrinolysis by plasmin generated by streptokinase or urokinase activation of purified plasminogen[9] was identical to that with spontaneously activated plasmin. The inhibition was also demonstrable by the fibrin plate method[17], in which non-radioactive fibrin is used as substrate (Fig. 3). Finally, addition of vitamin E to urokinase-stimulated normal plasma, at concentrations producing 50% inhibition of purified plasmin, resulted in 25% inhibition of ^{125}I-fibrin lysis.

Some observed effects of vitamin E esters, particularly the phosphate, which inhibits trypsin and papain[15], have been attributed to their anionic nature rather than to tocopheryl group[3], and may be mimicked (in the case of inhibition of hyaluronidase) by heparin and sodium dodecyl sulphate[16]. Neither of these agents, tested at 10^{-5} M, inhibited lysis of ^{125}I-fibrin by plasmin. Vitamin E did not inhibit lysis by four other proteolytic enzymes known to digest this substrate[5,8] (Table 1), at similar molar concentrations of the enzymes (maximal estimated concentrations, 0.5×10^{-7} M for collagenase to 3.3×10^{-7} M for trypsin). Additional evidence for the absence of an anionic effect has been provided by affinity chromatography experiments. D-α-tocopheryl succinate was coupled to aminohexyl Sepharose 4B (AH Sepharose, Pharmacia) by the carbodiimide reaction. With a column (1 ml bed volume) of this conjugate, in which the succinyl carboxyl group is blocked, application of purified plasmin (260 μg) resulted in binding of 80%, and subsequent elution of 60% of the bound plasmin by buffer containing D-α-tocopheryl succinate. Finally, inhibition of fibrinolysis by plasmin does not appear to be due to the ester form of the vitamin, since similar inhibition was produced by the free phenol. Pure D-α-tocopherol (Eastman Kodak), dissolved in ethanol because of its insolubility, was added to and incubated with plasmin (5 μg ml^{-1}, 30 min at 37 °C). Fifty per cent inhibition of plasmin lysis of ^{125}I-fibrin occurred at a free phenol concentration of 1.6×10^{-5} M, which compares with similar inhibition by the succinate ester at 0.6×10^{-5} M (Fig. 2). Lysis by plasmin plus ethanol (1.5% by volume) was 3,641 c.p.m., whereas lysis by plasmin in presence of D-α-tocopherol and ethanol was 1,855 c.p.m.

Reported levels of vitamin E in normal human plasma range from 6.5 to 15 μg ml^{-1} (1.6 to 3.3×10^{-5} M)[18], concentrations greater than those producing 50% inhibition of

Fig. 2 Effects of varying concentrations of vitamin E on lysis of ^{125}I-fibrin by plasmin (5 μg ml^{-1}). Assay conditions identical to those described in the legend to Fig. 1.



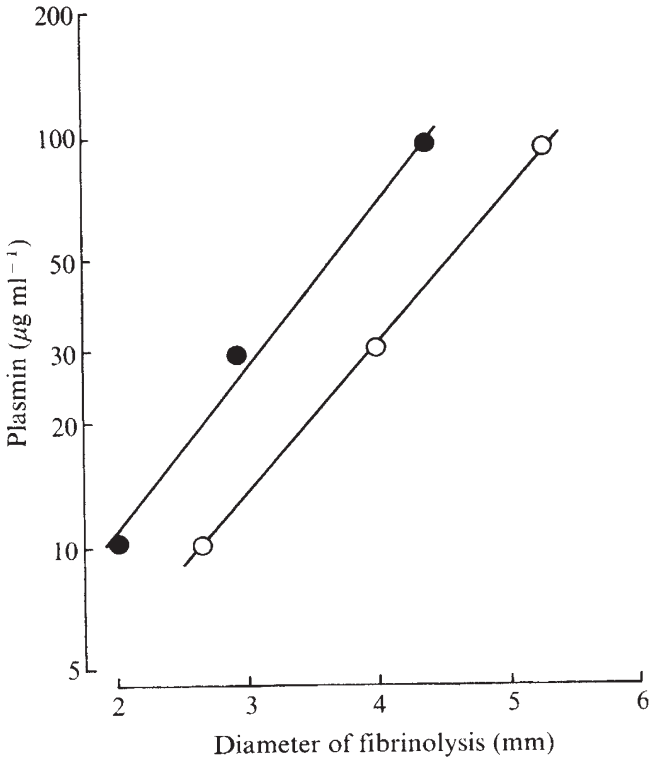


Fig. 3 Effect of vitamin E on lysis of fibrin by plasmin. Plasmin (10, 30 and 100 μg ml^{-1}), with or without vitamin E (1.5×10^{-5} M) was introduced into wells (5 μl per well) of heat-treated fibrin plates (Enzo-diffusion plates, Hyland). Diameter of lysis was measured after incubation at 37 °C for 6 h. Semilogarithmic plot plasmin concentration against diameter of lysis, with (●) and without (○) vitamin E. Operational zero point for the assay (no detectable lysis) is 2 mm, the diameter of the wells.

plasmin *in vitro* (Figs 1 and 2). Mean plasma levels of plasminogen-plasmin, determined immunochemically, are approximately 200 μg ml^{-1} (ref. 19). Although comprehensive data on relative proportions of proenzyme and active enzyme in normal plasma are lacking, it is generally accepted that almost all is in the proenzyme form[20]. In the present study, for example, plasminogen-plasmin, freshly prepared from normal plasma[8], released 360 c.p.m. from ^{125}I-fibrin, representing less than 2% of the activity (18,911 c.p.m. released) of the same preparation (enzyme concentration, 5 μg ml^{-1}) after maximal urokinase activation. This estimated plasmin concentration (2% of 200 μg ml^{-1}, or 4 μg ml^{-1}) is of the same order as the concentrations examined here, and agrees with the observations of others[21]. Relative to other inhibitors, vitamin E produces 50% inhibition at the same concentration (approximately 10^{-5} M) as EACA or tranexamic acid (*trans*-4-aminomethylcyclohexonecarboxylic acid)[8].

These observations may be pertinent to certain physiological and pathological phenomena, and to vitamin E therapy. Plasmin is normally inhibited by anti-plasmins in plasma. These include such macromolecules as α_2-macroglobulin, α_1-antitrypsin and C1 esterase inhibitor[22]. The inhibition of plasmin at physiological concentrations of vitamin E and enzyme raises the possibility that the vitamin may play a role in normal modulation of fibrinolysis. In addition, considering the broad substrate specificity of plasmin for many tissue proteins in addition to fibrin[5], this effect may be relevant to foetal resorption in vitamin E deficient animals (that is, unopposed proteolytic activity), which led to the initial recognition of this vitamin[23]. The *in vitro* inhibition of plasmin provides an explanation for the reported blunting, on oral vitamin E therapy, of the physiological increase in fibrinolytic activity after venous occlusion[7], a response considered to reflect increased conversion of plasminogen to plasmin, mediated by tissue activators released, presumably from vascular endothelium, in response to hypoxia[20]. Further, fibrinolysis has been implicated in the development and spread of tumours[24]. Both EACA and vitamin E inhibit fibrinolysis. Administration of EACA decreases metastatic spread of transplantable tumours in rabbits and rats[24], and there is a decreased incidence of carcinogen-induced lung tumours in vitamin E deficient mice when compared with normal mice, or deficient mice receiving vitamin E replacement[25]. Finally, in view of the widespread use of the vitamin and its succinate and acetate esters, and the observed effects of oral administration on fibrinolysis *in vivo*[7], the observations presented here may have implications for the consequences of dietary supplementation with the vitamin.

This work was supported by grants from the MRC of Canada and the Canadian Arthritis Society.

L. A. Moroz
N. J. Gilmore

The Harry Webster Thorp Laboratories,
Division of Clinical Immunology,
McGill University Clinic,
Royal Victoria Hospital,
Montreal, Quebec, Canada H3A 1A1

Received June 30; accepted October 27, 1975.

1 Molenaar, I., Vos, J., and Hommes, F. A., *Vitamin Horm.*, **30**, 45–82 (1972).
2 Marks, J., *Vitamin Horm.*, **32**, 131–154 (1974).
3 Olson, R. E., *Circulation*, **48**, 179–184 (1973).
4 Editorial, *Lancet*, **i**, 18 (1974).
5 Ratnoff, O. D., *Adv. Immun.*, **10**, 145–227 (1969).
6 Kay, J. H., Hutton, S. B., Jr, Weiss, G. N., and Ochsner, A., *Surgery*, **28**, 24–28 (1950).
7 Korsan-Bengtsen, K., Elmfeldt, D., and Holm, T., *Thromb. Diathes. haemorrh. (Stuttg.)*, **31**, 505–512 (1974).
8 Moroz, L. A., and Gilmore, N. J., *Blood*, **46**, 543–553 (1975).
9 Deutsch, D. G., and Mertz, E. T., *Science*, **170**, 1095–1096 (1970).
10 Brockway, W. J., and Castellino, F. J., *Archs Biochem.*, **151**, 194–199 (1972).
11 Robbins, K. C., Summaria, L., Elwyn, D., and Barlow, G. H., *J. biol. Chem.*, **240**, 541–550 (1965).
12 Lambertsen, G., and Braekkan, O., *Analyst*, **84**, 706–711 (1959).
13 Furter, M., and Meyer, R. E., *Helv. chim. Acta*, **22**, 240–250 (1939).
14 Robbins, K. C., and Summaria, L., *Ann. N.Y. Acad. Sci.*, **209**, 397–404 (1973).
15 Zierler, K. L., Grob, D., and Lilienthal, J. L., *Am. J. Physiol.*, **153**, 127–132 (1948).
16 Miller, W. H., and Dessert, A. M., *Ann. N.Y. Acad. Sci.*, **52**, 167–179 (1949).
17 Lassen, M., *Acta Physiol. scand.*, **27**, 371–376 (1952).
18 Oski, F. A., and Barness, L. A., *J. Pediat.*, **70**, 211–220 (1967).
19 Rabiner, S. F., Goldfine, I. D., Hart, A., Summaria, L., and Robbins, K. C., *J. lab. clin. Med.*, **74**, 265–273 (1969).
20 Sherry, S., *A. Rev. Med.*, **19**, 247–268 (1968).
21 Magoon, E. H., Austen, K. F., and Spragg, J., *Clin. exp. Immun.*, **17**, 345–359 (1974).
22 Evans, H. M., and Bishop, K. S., *J. metab. Res.*, **1**, 319–356 (1922).
23 Schreiber, A. D., Kaplan, A. P., and Austen, K. F., *J. clin. Invest.*, **52**, 1394–1401 (1973).
24 Clifton, E. E., *Fedn Proc.*, **25**, 89–93 (1966).
25 Telford, I. R., *Ann. N.Y. Acad. Sci.*, **52**, 132–134 (1949).

Confusion between specific and nonspecific binding of carcinoembryonic antigen and blood-group antigens by eluted antibody preparations

Cross reactivity between carcinoembryonic antigen (CEA) and blood-group antigens is inferred from the observation that many human sera can bind labelled CEA. This is more common with sera of blood group O or B (that is containing anti-A antibodies), and binding may sometimes be inhibited by absorption of the sera with group A erythrocytes[1,2].

Following up these observations, Holburn *et al.*[3] showed that hyperimmune human antisera against A, B, Le^a and Le^b antigens could bind 30–75% of added labelled CEA. They concluded that the extent of binding suggested that both CEA and blood-group antigens were present on the same molecule. Freedman, commenting on these experiments, pointed out that a hyperimmune antiserum will contain many other "sticky" substances as well as antibodies, and these might account for the results (paper read at meeting of British Society for Immunology, autumn,