Biochem. J. (1984) **220**, 625–630
Printed in Great Britain

An inverse relationship between heparin content and antibody response in genetically selected mice

Sex effect and evidence of a polygenic control for skin heparin concentration

Anita H. STRAUS,* Osvaldo A. SANT'ANNA,† Helena B. NADER* and Carl P. DIETRICH*
**Departamento de Bioquímica, Escola Paulista de Medicina, C.P. 20372, São Paulo, SP, Brazil, and*
†Seção de Imunologia, Instituto Biológico, C.P. 7119, São Paulo, SP, Brazil

(*Received 27 September 1983/Accepted 21 February 1984*)

The heparin content of genetically selected mice with high and low antibody response to bacterial antigens is reported. An inverse relationship between antibody titres and concentration of heparin was observed for both male and female mice. The lower-antibody-responder line contains twice as much heparin as the higher-responder ones. Furthermore, the female mice also contained twice as much heparin as the male mice. Genetic analysis of the parental and interline hybrids has shown a partial dominance for the character 'heparin content' in favour of the high-heparin phenotype and this character appears to be subjected to polygenic control. The possible biological role of heparin and/or mast cells in the surveillance of the organism against some pathogens is discussed in the light of these and other findings.

Heparin has been widely used in medicine on account of its well-known pharmacological activities as an anticoagulant (Jorpes, 1946), antilipaemic (Levy, 1958) and antihaemostatic agent (Cruz & Dietrich, 1967). Besides, several other actions have been attributed to heparin, such as inhibition of several enzymes, among them myosin ATPase (Cruz & Dietrich, 1967), RNA-dependent DNA polymerase (Newhoff *et al.*, 1970), hyaluronidase, elastase and renin (Sealey, 1967), effects on tumour growth (Lippman, 1957), antibacterial activity (Corrigan, 1977) and antiviral action (Vaheri, 1964). It has also been suggested that heparin could function as a local anticoagulant, owing to the distribution of mast cells in the vicinity of small vessels (Jaques, 1967). The presence of heparin in mast cells led also to the suggestion that heparin could function as a trapper for biogenic amines (Padawer, 1974; Uvnäs, 1979). In spite of all this evidence, its actual biological role remains unknown.

A recent survey on the distribution of heparin in twelve tissues of nine mammalian species (Nader *et al.*, 1980) and previously reported data (Barlow *et al.*, 1964; Toledo & Dietrich, 1977) has shown that heparin was present in relatively high amounts in lung, ileum and skin of most species analysed. Conversely, except for thymus and lymph nodes, none or small amounts of heparin were detected in all other tissues. From the several novel observations regarding the distribution of heparin, the most peculiar ones were the presence of this compound in relatively large amounts in lymphoid tissues, its unequivocal presence in humans and its absence in rabbit tissues. The study on the distribution of heparin during foetal development has shown that heparin is present almost exclusively in the haematopoietic and lymphoid tissues of foetuses (spleen, thymus and lymph nodes), whereas ileum, skin and lung, besides lymph nodes and bovine thymus, are heparin-rich tissues in adult animals (Nader *et al.*, 1982). A good correlation between the concentration of heparin and the numbers of mast cells was observed in all tissues examined (Straus *et al.*, 1982*a*,*b*). The peculiar distribution of heparin in mammal tissues, the small amount of heparin in foetal tissues, its decrease in liver and spleen, its appearance in skin, ileum and lung, tissues directly exposed to the environment after birth, its preferential localization at the maternal side of the placenta, together with other data, led to the suggestion that heparin may be involved with defence mechanisms either directly or through the immune response, as has been previously speculated (Jaques, 1967).

Siqueira *et al.* (1976) have selected high (H)- and low (L)- responder lines of mice according to the maximal or minimal antibody production by a two-

way selective process against the flagellar antigens (Agf) of salmonellae (Selection III). After sixteen generations of the selective breeding, H and L lines were considered homozygous for the relevant alleles that control this quantitative character (Siqueira *et al.*, 1976). In this selection the character is controlled by about seven independent loci (Sant'Anna *et al.*, 1982). The response to Agf in terms of agglutinin titres is 1:7100 in H/f and 1:90 in L/f mice. When tested with a large variety of antigens, unrelated to those used during selection, H and L lines were found to produce high and low antibody levels. These findings clearly show the non-specific effect of the accumulated H and L genes (Siqueira *et al.*, 1977; Biozzi *et al.*, 1979). Thus these lines of mice seemed to be an interesting model to test the above-mentioned hypothesis concerning the involvement of heparin with immune humoral response.

The present paper describes significant differences of heparin content between two selected high- and low-responder lines as well as a significant sex difference regarding the concentration of heparin in tissues.

Experimental procedures

Mice

High (H/f)- and low (L/f)-responder lines of mice from the F_{27}–F_{32} generations of Selection III were analysed when 2–4 months old. Reciprocal interline crosses: F_1 (H/f × L/f) and F_2 ($F_1 \times F_1$) were bred from the F_{30} generation of the parental H/f and L/f lines.

Immunization and agglutinin assay

Mice were immunized with two injections containing 3.3×10^8 *Salmonella typhimurium* cells, 8 days apart. The f (flagellar) agglutinin titres were measured by direct agglutination in individual serum samples as previously described and expressed as $\log_2$ of the highest dilution giving a positive agglutination (Siqueira *et al.*, 1976).

Materials and enzymes

Chondroitin 4- and 6-sulphates, dermatan sulphate and chondroitinases AC and ABC were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Heparin was kindly given by Dr. M. B. Mathews (University of Chicago, Chicago, IL, U.S.A.), LAOB Laboratories (São Paulo, Brazil), INORP Laboratories (Buenos Aires, Argentina) and Upjohn Co. (Kalamazoo, MI, U.S.A.). Heparan sulphate, heparinase and heparitinases were prepared by methods described previously (Dietrich & Nader, 1974; Silva & Dietrich, 1975). Agarose was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). 1,3-Diaminopropane was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Superase (proteolytic enzyme from a strain of spore-forming bacillus) was purchased from Chas. Pfizer and Co. (New York, NY, U.S.A.).

Extraction of heparin and other sulphated glycosaminoglycans

The tissues were ground with 10 vol. of acetone and after being left overnight at 4°C the mixture was centrifuged (3000 ***g***/10 min), washed once with acetone and dried under vacuum. Heparin was extracted from the tissues by a procedure (Toledo & Dietrich, 1977) modified from that described by Scott *et al.* (1968) as follows: 0.5 g of dry tissue was suspended in 10 ml of 0.05 M-Tris/HCl buffer, pH 8.0, in the presence of 0.15 M-NaCl. The suspension was incubated at 37°C for 24 h with 20 mg of superase and a few drops of toluene. After the incubation, 1.98 g of the anhydrous potassium acetate were added to the incubation and the mixture shaken to insure complete solution of the salt. The pH was adjusted to 5.7 and the suspension maintained in an oven for 2 h at 60°C. About 100 mg of Celite was added to the suspension, which was then filtered at 60°C. The filtrate was collected in a centrifuge tube and kept at 4°C overnight. The precipitate was collected by centrifugation (5000 ***g***/120 min) in the cold and dissolved in 0.5 ml of water. Ethanol (2 vol.) was added to this solution. After being left overnight in the cold the precipitate formed was collected by centrifugation (5000 ***g***/30 min) and dried. It was then dissolved in 200 μl of water and analysed for heparin. To the supernatant remaining after precipitation of heparin in the cold, 2 vol. of ethanol was added. The precipitate formed was collected by centrifugation (5000 ***g***/30 min), dried and resuspended in 300 μl of water and analysed. The recovery of sulphated glycosaminoglycans by this procedure was comparable with the method used previously (Dietrich *et al.*, 1976). Furthermore, this method had the advantage of separating heparin from the other sulphated glycosaminoglycans. Recovery experiments with heparin and the other sulphated glycosaminoglycans have shown that only heparin precipitates with potassium acetate and all the other sulphated glycosaminoglycans remain in the supernatant, free of heparin. In order to assess the reproducibility of the method, seven extractions of the skin of the same mice were performed. The results obtained (605 ± 38 μg/g of dry tissue) gave a variation coefficient of 6%. The limit of detection of heparin by the use of this procedure was 0.5 μg/g of dry tissue.

Identification and quantification of heparin and other sulphated glycosaminoglycans

Heparin and other sulphated glycosaminoglycans were identified by a combination of agarose-gel electrophoresis and enzymic degradation with specific enzymes as previously described (Dietrich & Dietrich, 1972, 1976; Bianchini *et al.*, 1980). The United States Pharmacopeia anticoagulant activity assay was also used to identify heparin. Heparin was quantified by the carbazole reaction (DiFerrante *et al.*, 1971). The other sulphated glycosaminoglycans were quantified by densitometry of the agarose slides after Toluidine Blue staining. The error of the method was in the order of $\pm 4.5\%$. The absorption coefficients of the glycosaminoglycans were calculated by using as standards chondroitin 4-sulphate, dermatan sulphate and heparan sulphate. Paper chromatography of the products formed after enzymic degradation was performed in isobutyric acid/1 M-NH_3 (5:3, v/v). M_r determinations were performed by polyacrylamide-gel electrophoresis (Dietrich & Nader, 1974).

Statistics

Significance was assessed by Student's t test. The best fit of correlation between antibody level and the amount of heparin was determined from a least-squares linear regression. The degree of association between the two variables is measured by the correlation coefficient. The genetic analysis is based on means of the $\log_2$ of heparin concentration of the H/f and L/f parental lines and their F_1 hybrids. The dominance effect (d/a) was determined by the global dominance (d) and additive effect (a) values as follows:

$$d = \bar{x}\,F_1 - \frac{\bar{x}\mathrm{H/f} + \bar{x}\mathrm{L/f}}{2}$$

$$a = \frac{\bar{x}\mathrm{H/f} - \bar{x}\mathrm{L/f}}{2}$$

Results

Concentration of heparin and other sulphated glycosaminoglycans in the H/f and L/f responder lines selected against flagellar antigens of salmonellae

Table 1 shows the amount of heparin present in the skin of the H/f and L/f responder lines. A significant difference ($P<0.001$) in the amount of heparin between the H/f and L/f lines was observed. The L/f mice have twice as much heparin as the H/f mice. Besides a significant difference between the two lines, there is also a significant difference ($P<0.001$) related to sex, regardless of the line studied. Females show about twice as much heparin as males. Other tissues contain smaller amounts of heparin (Straus *et al.*, 1982*a*) and have not been analysed. Table 2 shows the concentration of the other sulphated glycosaminoglycans in the skin of females and males of the two lines. No variation of heparan sulphate concentration could be observed between sex and the L/f and H/f responder lines, contrasting with dermatan sulphate, where a 14–25% variation was observed between the L/f and H/f lines as well as between males and females.

Heparin concentration in $F_1(H/f \times L/f)$ and F_2 $(F_1 \times F_1)$ hybrids

Table 3 shows the results of the heparin concentration in the F_1 (H/f × L/f) and F_2 ($F_1 \times F_1$) interline hybrids. In F_1 as well as in F_2 hybrids the heparin concentration was not significantly different from the L/f parental line. On the other hand, the same pattern of sex difference shown for the H/f and L/f mice was also observed in the F_1 offspring. Although the variance values of the F_2 female population had been highest, no difference was observed between F_1 and F_2 regarding the mean heparin concentration. Fig. 1 expresses the $\log_2$ distribution of the heparin concentration in females of both parental lines and their F_1 and F_2 hybrids. The dominance effect of the character 'heparin concentration' was found to

Table 1. *Heparin concentration in skin of H/f and L/f lines selected to flagellar antigens (Agf) of salmonellae*
The differences between the sexes is statistically significant ($P<0.001$). The results are means ± S.D.; the variance (v) is also shown.

		Females			Males		
Line	$\log_2$ (anti-Agf titre)	No. of mice	Heparin (μg/g of dry tissue)		No. of mice	Heparin (μg/g of dry tissue)	
L/f	6.5	11	966 ± 223 $v = 49729$	$P<0.001$	16	442 ± 84 $v = 7056$	$P<0.001$
H/f	12.8	10	494 ± 84 $v = 7056$		15	240 ± 61 $v = 3721$	

Table 2. *Sulphated-glycosaminoglycan concentration in skin of H/f and L/f lines selected to flagellar antigens of salmonellae*

	Concentration (μg/g of dry tissue)					
	Females			Males		
Line	Heparin	Dermatan sulphate	Heparan sulphate	Heparin	Dermatan sulphate	Heparan sulphate
L/f	966±223	783±72	106±19	442±84	907±86	97±17
H/f	494±84	587±106	106±61	240±61	766±71	99±19

Table 3. *Heparin concentration in the F_1 (H/f × L/f) and F_2(F_1 × F_1) interline hybrids*
Results are means±S.D. Abbreviations used: *v*, variance; n.s., not significant. The differences between the sexes is statistically significant ($P<0.001$).

		Females		Males	
Line	$\log_2$(anti-Agf titre)	No. of mice	Heparin (μg/g of dry tissue)	No. of mice	Heparin (μg/g of dry tissue)
F_1 (H/f × L/f)	10.1	18	792±159 *v* = 25281	12	419±104 *v* = 10816
F_2 (F_1 × F_1)	10.7	45	880±202 *v* = 40804		n.s.

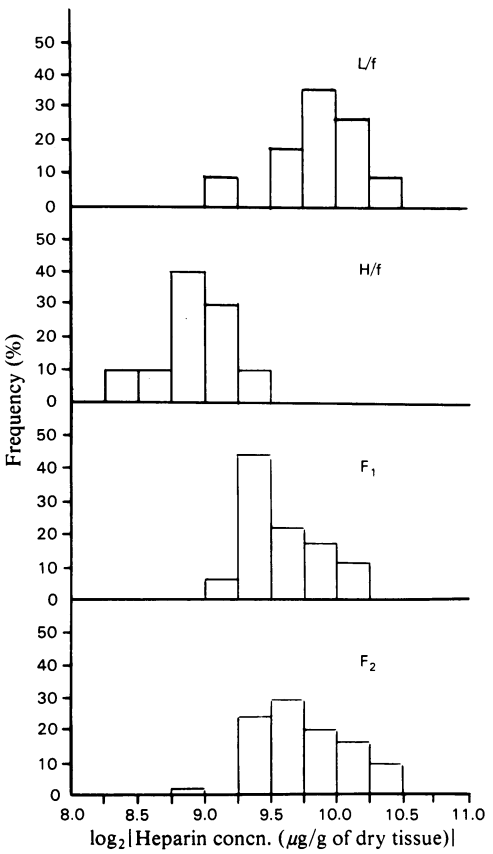

Fig. 1. *Frequency distribution of heparin concentration in females of parental lines (L/f and H/f) and their F_1 and F_2 hybrids*

be about 50% of the high heparin tissue content expressed in the L/f line ($d/a = -0.48$).

The heparin content and antibody production in the immunized F_2 population was analysed. It was shown that there was no significant inverse correlation (−0.20) in most of the F_2 mice.

Chemical and physico-chemical characteristics and anticoagulant activity of the heparins extracted from the skin of the H/f and L/f lines

Fig. 2 shows the electrophoretic behaviour, in two different buffer systems, of the heparins extracted from the skin of the H/f and L/f lines as well as from the F_1 (H/f × L/f) according to sex. In the discontinuous system, barium acetate/1,3-diaminopropane (Fig. 2*a*), heparins from different sources have a lower electrophoretic migration when compared with the other sulphated glycosaminoglycans. The mice heparins also show the formation of multiple bands in this system as well as in diaminopropane/acetate buffer (Fig. 2*b*). This appears to be related to their macromolecular nature, since it has been previously observed in several other mice heparins such as those of the 'nude', Balb and other strains (Straus *et al.*, 1982*a*) as well as in rat heparins (Nader *et al.*, 1980; Horner, 1971). Furthermore, these heparins, when degraded by the heparinase from *Flavobacterium heparinum*, formed the typical products of a heparin preparation, that is, pentasulphated tetrasaccharide and trisulphated disaccharide (Silva & Dietrich, 1975; Nader *et al.*, 1982; Straus *et al.*, 1982*a*,*b*). No significant differences could be observed between the heparins obtained from H/f

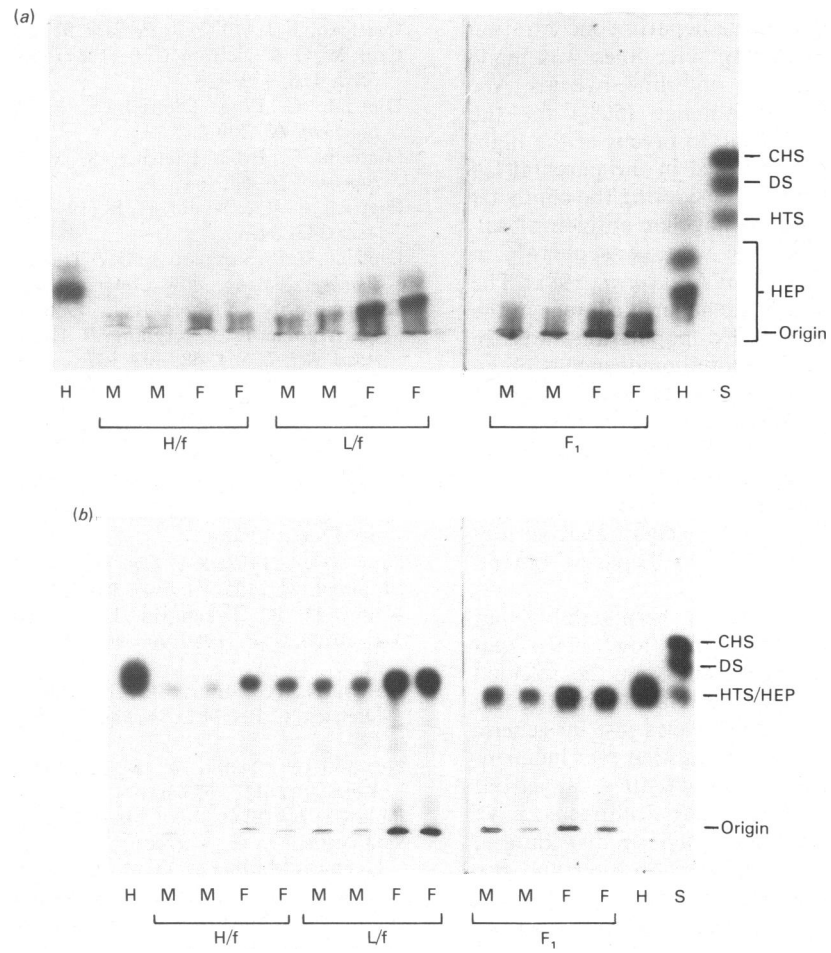

Fig. 2. *Electrophoretic behaviour of heparins extracted from skin of H/f and L/f lines and their F_1 hybrids*
(*a*) Portions (5 μl) of heparins obtained by the potassium acetate procedure were applied on agarose-gel slabs prepared in 0.04 M-barium acetate, pH 5.8, and subjected to electrophoresis (10 min, 75 V) in a chamber prepared with the same buffer at 5°C. The whole gel slabs were then transferred to another chamber, prepared with the diaminopropane/acetate buffer and maintained at 5°C for 15 min. The current (100 V) was then applied in the same direction (towards the positive electrode) for 90 min. The heparins were fixed in the gel with Cetavlon (cetyltrimethylammonium bromide) and stained with Toluidine Blue. (*b*) Portions (5 μl) of heparins were applied on agarose-gel slabs prepared with 0.05 M-1,3-diaminopropane/acetate buffer, pH 9.0, and subjected to electrophoresis for 1 h at 120 V. The gels were fixed and stained as described in (*a*). Abbreviations used: M, male; F, female; H, HEP, commercial heparin; S, sulphated glycosaminoglycans [standard mixture containing chondroitin 4- and 6-sulphates (CHS), dermatan sulphate (DS) and heparan sulphate (HTS).

and L/f lines regarding the anticoagulant activity (100–120 i.u./mg) and M_r (>150000).

Discussion

The present results indicate that low-antibody-responder mice contain twice as much heparin as the high-antibody responders. These lines were selected according to the maximal or minimal antibody production against flagellar salmonellae antigens. Another independent genetic selection (selection IV) (Siqueira *et al.*, 1976) obtained with the somatic salmonellae antigens gave essentially the same co-relationship, i.e. the low-responder mice had from 1.5 to 2.0 times as much heparin as the corresponding high-responder line. No exception to this pattern was found in both selections in more than 60 animals analysed.

Another striking feature of these analyses were the findings concerning the heparin content in different sexes. Thus females had twice as high a mean heparin concentration as males.

A genetic analysis of the heparin concentration in the low- and high-responder lines was made through the F_1 and F_2 interline hybrids. We observed a partial dominance (50%) for the character 'heparin content' in favour of the high-heparin phenotype expressed in the parental L/f line. On the other hand, considering the character 'antibody production' to flagellar antigen of salmonellae, the dominance value was of 14% in favour of the high line (Sant'Anna *et al.*, 1982). The phenotypic variance of the heparin content in the heterogeneous genetic F_2 population was in general higher than for those homogeneous populations of L/f, H/f and F_1 mice. The only exception to these observations was noted in the females of the L/f line. For both parental lines and offspring (F_1 and F_2) mice a normal distribution was observed for the $\log_2$(heparin concentration) (Fig. 1). These combined results on the genetic analysis suggest polygenic control for the 'heparin content' character.

None of these data rule out the possibility that the accumulation of 'high' and 'low' alleles that control antibody responsiveness in the selected high and low lines could be related with the low and high 'heparin content' alleles just by genetic drift. Nevertheless, the finding that two independent genetic selections of H and L mice, carried out against two non-related bacterial antigens, have also shown the same type of heparin distribution, suggest that the heparin concentration and the level of antibody response do not occur by chance. This is also in accordance with the already-mentioned suggestion that heparin may function as an alternative mechanism for the surveillance of the organism against some pathogens (Nader *et al.*, 1980, 1982; Straus *et al.*, 1982*b*).

This work was aided by grants from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), FINEP (Financiadora de Estudos e Projetos), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and PIG IV 40239/82.

References

Barlow, G. H., Coen, L. J. & Mozen, M. M. (1964) *Biochim. Biophys. Acta* **83**, 272–277

Bianchini, P., Nader, H. B., Takahashi, H. K., Osima, B., Straus, A. H. & Dietrich, C. P. (1980) *J. Chromatogr.* **196**, 455–462

Biozzi, G., Monton, D., Sant'Anna, O. A., Passos, M. C., Gennari, M., Reis, M. H., Ferreira, V. C. A., Hermann, A. M., Bonthillier, Y., Ibañez, O. M., Stiffel, C. & Siqueira, M. (1979) *Curr. Top. Microbiol. Immunol.* **85**, 31–98

Corrigan, J. J. (1977) *J. Pediatr.* **91**, 695–700

Cruz, W. O. & Dietrich, C. P. (1967) *Proc. Soc. Exp. Biol. Med.* **126**, 420–426

Dietrich, C. P. & Dietrich, S. M. C. (1972) *Anal. Biochem.* **46**, 209–215

Dietrich, C. P. & Dietrich, S. M. C. (1976) *Anal. Biochem.* **70**, 645–647

Dietrich, C. P. & Nader, H. B. (1974) *Biochim. Biophys. Acta* **343**, 34–44

Dietrich, C. P., Sampaio, L. O. & Toledo, O. M. S. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1–10

DiFerrante, N., Nichols, B. L., Donnelly, P. V., Neri, G., Argovic, R. & Berglung, R. K. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 303–307

Horner, A. (1971) *J. Biol. Chem.* **246**, 231–239

Jaques, L. B. (1967) in *Progress in Medicinal Chemistry* (Ellis, G. P. & West, G. B., eds.), pp. 139–198, Butterworths, London

Jorpes, J. E. (1946) in *Heparin: Its Chemistry, Physiology and Application in Medicine*, 2nd Edn., Oxford University Press, London

Levy, S. W. (1958) *Rev. Can. Biol.* **17**, 1–61

Lippman, M. (1957) *Cancer Res.* **17**, 11–14

Nader, H. B., Takahashi, H. K., Straus, A. H. & Dietrich, C. P. (1980) *Biochim. Biophys. Acta* **627**, 40–48

Nader, H. B., Straus, A. H., Takahashi, H. K. & Dietrich, C. P. (1982) *Biochim. Biophys. Acta* **714**, 292–297

Newhoff, V., Schill, W. B. & Sternbach, H. (1970) *Biochem. J.* **117**, 623–631

Padawer, J. (1974) *Am. J. Anat.* **141**, 299–302

Sant'Anna, O. A., Ferreira, V. C. A., Reis, M. H., Gennari, M., Ibañez, O. M., Esteves, M. B., Mouton, D. & Biozzi, G. (1982) *J. Immunogenet.* **9**, 191–205

Scott, J. E., Stacey, T. E. & Tigwell, M. J. (1968) *Biochem. J.* **108**, 50P

Sealey, J. E., Gerten, J. N., Ledingham, J. G. G. & Laragh, J. H. (1967) *J. Clin. Endocrinol. Metab.* **27**, 699–705

Silva, M. E. & Dietrich, C. P. (1975) *J. Biol. Chem.* **250**, 6841–6846

Siqueira, M., Bandieri, A., Reis, M. S., Sant'Anna, O. A. & Biozzi, G. (1976) *Eur. J. Immunol.* **6**, 241–249

Siqueira, M., Esteves, M. B., Ibañez, O. M., Ferreira, V. C. A., Sant'Anna, O. A., Reis, M. H. & Biozzi, G. (1977) *Eur. J. Immunol.* **7**, 195–203

Straus, A. H., Nader, H. B. & Dietrich, C. P. (1982*a*) *Biochim. Biophys. Acta* **717**, 478–485

Straus, A. H., Nader, H. B., Takahashi, H. K. & Dietrich, C. P. (1982*b*) *An Acad. Bras. Cienc.* **54**, 439–448

Toledo, O. M. S. & Dietrich, C. P. (1977) *Biochim. Biophys. Acta* **498**, 114–122

Uvnäs, B. (1979) in *Heparin: Structure, Cellular Function and Clinical Application* (McDuffie, N. M., ed.), pp. 243–250, Academic Press, New York

Vaheri, A. (1964) *Acta Pathol. Microbiol. Scand. Suppl.* **171**, 7–98