Lymphoid Organs and Normal Gamma-Globulin in the Rat

Abstract. *Serum gamma-globulin was determined in adult rats several months after total thymectomy, subtotal splenectomy, or a combination of total thymectomy, subtotal splenectomy, and subtotal lymphadenectomy. The thymus apparently is of little importance in the production of normal gamma-globulin, but it may influence the production of albumin because a reduction of serum albumin was only seen in groups where thymectomy was performed. The spleen seems to produce normal gamma-globulin to a greater extent than lymphoid tissue elsewhere in the body.*

It is widely accepted that antibody γ-globulin formation takes place predominantly in the plasmocytic cell lines (*1*), and normal γ-globulin is also formed by these cells (*2*). Plasma cells usually occur in the bone marrow, liver, spleen, thymus, and the lymph nodes as well as in the lymphoid tissues of the intestinal tract. The spleen and other lymphoid tissues seem to play a major role in antibody formation during immunization (*3*) but apparently antibodies are also formed in the lungs (*4*). Normal serum γ-globulin may consist of antibodies exclusively but this question has not been finally settled. Normal γ-globulin is probably formed by the same organs that form antibody γ-globulin, although it is not known which organs or organ systems are most important as sites of formation of normal γ-globulin. Recent experiments have shown that thymectomy shortly after birth may inhibit the normal development of the immunological capacity of the organism (*5*), and it is known that patients with thymoma may develop agammaglobulinemia (*6*). Therefore, we have studied the role of the thymus and other lymphoid organs in adult rats for the production of normal γ-globulin.

Paper electrophoresis (*7*) was used to determine the serum γ-globulin in groups of inbred young adult female albino rats. This was done several months after various surgical procedures (weight at the time of operation was about 75 grams; age, 6 weeks). Total thymectomy was performed in ten rats. Subtotal splenectomy (80 to 90 percent) was performed in ten other rats. Subtotal splenectomy was performed instead of total splenectomy in order to avoid *Bartonella muris* infection. Seven rats were subjected to a combination of total thymectomy, subtotal splenectomy, and removal of all macroscopically visible abdominal, epigastric, brachial, axillary, and deep and superficial cervical lymph nodes. A sham control operation was performed in ten rats, and ten rats served as unoperated controls. All surgery was carried out under ether anesthesia. The procedure has been described in detail by Bierring (*8*). All operated animals recovered quickly and gained weight at the same rate as the unoperated controls. The animals were killed by bleeding through an incision of the abdominal aorta 65 or 90 days after the operations. In no case were remnants of the thymus found in thymectomized rats. Earlier experiments have shown that the number of lymph nodes was reduced an average of 68 percent by the extirpation technique that we used (*8*).

Table 1 gives the average results of the paper electrophoretic analysis in the various groups. The concentration of γ-globulin in the control group and in the sham-operated group was almost identical, but since the concentration of β-globulin was higher in the sham-operated animals ($p < 0.05$), this group was used as the reference for the other groups.

Total thymectomy did not alter the electrophoretic pattern significantly although there was a slight decrease in albumin. Subtotal splenectomy caused a highly significant decrease in γ-globulin as well as in β-globulin ($p < 0.001$), and a slight decrease in α_1-globulin ($p < 0.05$). Combined total thymectomy, subtotal splenectomy, and subtotal lymphadenectomy gave the most pronounced deviation from the results in the sham-operated group. A highly significant decrease was seen in γ-globulin and in β-globulin ($p < 0.001$), but a fall was also seen in the albumin fraction ($p < 0.05$) and in the α_1-globulin fraction ($p < 0.02$).

These results suggest that the thymus is of minor importance in the production of γ-globulin in the normal adult rat. Rather surprisingly it was found that the thymus may influence the concentration of albumin, because a reduction of serum albumin was only seen in groups where thymectomy was performed. Albumin is synthesized in the liver and it is difficult to imagine a thymic influence on the liver except by humoral factors. On the other hand, several factors other than production, such as an increase in the plasma volume, may influence the serum concentration.

As already mentioned, the spleen accounts for a substantial part of the production of antibodies. The present results indicate that the spleen is important for the production of normal γ-globulin also. The concentration of γ-globulin was not further reduced by subtotal lymphadenectomy, although it is probable that more lymphoid tissue was removed by this method

Table 1. Average results of paper electrophoresis in adult rats after thymectomy, or splenectomy, or combined splenectomy, thymectomy, and subtotal lymphadenectomy. The results are given in grams per 100 ml, with the standard error of the mean. N = number of rats in each group.

Total serum protein (Biureth)	Serum albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
		Control animals ($N = 10$)			
6.82 ± 0.097	4.21 ± 0.114	0.51 ±0.037	0.57 ± 0.020	0.88 ± 0.040	0.70 ± 0.031
		Sham-operated controls ($N = 10$)			
7.06 ± 0.058	4.20 ± 0.038	0.52 ± 0.030	0.53 ± 0.045	1.07 ± 0.069	0.74 ± 0.040
		Total thymectomy ($N = 10$)			
6.96 ± 0.091	4.07 ± 0.150	0.49 ± 0.031	0.57 ± 0.022	1.05 ± 0.052	0.76 ± 0.046
		Subtotal splenectomy ($N = 10$)			
6.39 ± 0.097	4.17 ± 0.081	0.45 ± 0.014	0.55 ± 0.011	0.76 ± 0.021	0.44 ± 0.018
		Total thymectomy, subtotal splenectomy, and subtotal lymphadenectomy ($N = 7$)			
6.20 ± 0.071	4.08 ± 0.049	0.40 ± 0.042	0.52 ± 0.022	0.72 ± 0.018	0.44 ± 0.028

than by subtotal splenectomy. The spleen, therefore, may be of greater importance in the production of normal γ-globulin than lymphoid tissue in other parts of the body.

A study by Andreasen and coworkers (*9*) is not in accordance with our investigation. They found that the serum protein fractions were within normal range after subtotal extirpation of lymphoid organs, including the spleen. The time of observation, however, was much shorter in their study than in ours. This may explain the discrepancy in results.

STIG BRYDE ANDERSEN*
FRANZ BIERRING
Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, and *Medicinsk-Anatomisk Institut, University of Copenhagen, Copenhagen, Denmark*

References and Notes

1. B. Bjørneboe and H. Gormsen, *Acta Pathol. Microbiol. Scand.* **20**, 649 (1943); A. Fagraeus, *Acta Med. Scand., Suppl.* **201**, 1 (1948); G. J. V. Nossal, *Brit. J. Exptl. Pathol.* **40**, 301 (1959).
2. E. H. Leduc, A. H. Coons, J. M. Conolly, *J. Exptl. Med.* **102**, 61 (1955); R. Asofsky and G. J. Thorbecke, *ibid.* **114**, 471 (1961); R. C. Mellors and L. Korngold, *ibid.* **118**, 387 (1963).
3. A. Fagraeus, *J. Immunol.* **58**, 1 (1948); G. J. Thorbecke and F. J. Keuning, *ibid.* **70**, 129 (1953); G. J. Thorbecke, R. M. Asofsky, G. M. Hochwald, G. W. Siskind, *J. Exptl. Med.* **116**, 295 (1962).
4. J. H. Humphrey and B. D. Sulitzeanu, *Biochem. J.* **68**, 146 (1958); B. A. Askanos and J. H. Humphrey, *ibid.* **70**, 212 (1958).
5. J. F. A. P. Miller, *Lancet* **1961-II**, 748 (1961); B. H. Waksman, B. G. Arnason, B. D. Janović, *J. Exptl. Med.* **116**, 187 (1962).
6. R. A. Good *et al.*, in *Progress in Allergy*, P. Kallos and B. H. Waksman, Eds. (Karger, Basel, Switzerland, 1962), vol. 6, p. 187.
7. C. B. Laurell, S. Laurell, N. Skoog, *Clin. Chem.* **2**, 99 (1956).
8. F. Bierring, *Acta Anat.* **50**, 64 (1962).
9. E. Andreasen, J. Bing, O. Gottlieb, N. Harboe, *Acta. Physiol. Scand.* **15**, 254 (1948).

* Present address: Department of Clinical Physiology, Glostrup Hospital, Glostrup (Copenhagen), Denmark.

19 March 1964

Intermolecular Cross-Linking of Collagen and the Identification of a New Beta-Component

Abstract. *Extraction of skin with 5M guanidine after salt and acid extraction yields a gelatin fraction which contains a greater proportion of double-chain (β) components than can be accounted for by intramolecular cross-linking of collagen molecules. This fraction also contains a new β-component, identified as the dimer of α2 and designated β_{22}. This dimer must be formed by intermolecular cross-linking since each collagen molecule contains only one α2 chain. Thus, direct evidence is presented for the occurrence of both inter- and intramolecular cross-linking by what appears to be a single continuous process.*

Chromatographic studies of denatured collagen show that the collagen monomer contains three chains, two of which (the α1 chains) are apparently identical while one (the α2 chain) is different in amino acid composition (*1, 2*). With time, covalent cross-links form between the chains to produce β-components (β_{12} is a covalently bonded dimer of the α1 and α2 chains; similarly β_{11} is a dimer of two α1 chains) (*3*). The rate and extent to which this occurs is dependent on the tissue and species (*2*).

Table 1. Partial amino acid composition of the component β_{22} compared with that of α1 and α2 chains in human skin collagen.

Amino acid	Residues per 1000 residues		
	α1	α2	β_{22}
Hydroxyproline	91	82	83
Proline	135	120	118
Valine	21	33	31
Isoleucine	7	15	14
Leucine	20	30	31
Histidine	2	10	11

It is believed that cross-linking is of importance to the structure and function of connective tissue since in at least one known pathological condition, lathyrism (*4*), interference with cross-linking results in a profound disorder of connective tissue. However, chromatographic studies reported thus far relate directly only to intramolecular cross-linking whereas it would appear that the process of intermolecular cross-linking is the one which imparts to collagen fibers properties important to the function of connective tissues. It has been necessary to assume, in the physiologic interpretation of these studies, that the two processes are related.

We present evidence that the process of cross-linking in skin collagen results in the formation of intermolecularly bonded double-chain components (and presumably, later, higher aggregates) which are identical or analogous to those resulting from intramolecular cross-linking.

Skin from several mammalian species was extracted at 5°C with 1*M* sodium chloride and then five times with 0.5*M* acetic acid to remove most of the extractable collagen. The residue was then extracted with 5*M* guanidine, *p*H 7.5, at 5°C for 24 hours. After clarification by filtration, the guanidine extract was dialyzed exhaustively against water and the resulting precipitate was lyophilized as a suspension. The results of hydroxyproline analysis indicated that the preparation contained about 75 percent collagen that was present as gelatin as a result of the denaturing action of guanidine (*5*). In the rat this fraction represented about 20 percent of the total skin collagen. Samples of the lyophilized material were dissolved by warming at 45°C for a few minutes in potassium acetate buffer (0.075 ionic strength), *p*H 4.8, for ultracentrifugation, or in sodium acetate buffer (0.06 ionic strength), *p*H 4.8, for chromatography (*2*). The samples did not dissolve completely but hydroxyproline analysis showed that most of the insoluble protein was noncollagenous.

Sedimentation velocity patterns in the ultracentrifuge demonstrated that the guanidine-extracted gelatin consisted largely of double-chain β-components. Small amounts of the α- and the triple-chain γ-components were also present (Fig. 1). For comparison a sample of denatured acid-extracted collagen containing about 50 percent β-component is included. In these patterns the ratio of β- to α-components appears to be smaller than it actually is because of the effect described by Johnston and Ogston (*6*).

The presence of large amounts of β-component was confirmed by chro-

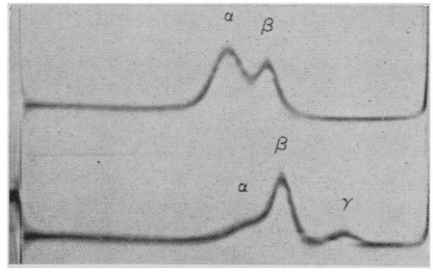

Fig. 1. Sedimentation patterns of heat-denatured collagen from human skin extracted with acid (upper pattern, wedged cell) and then extracted with 5*M* guanidine (lower pattern, standard cell). Centrifugation performed in 12-mm cells at 59,780 rev/min in 0.15*M* potassium acetate at *p*H 4.8 and 40°C; phase plate angle 65°C, sedimentation from left to right, exposure at 106 minutes.