No measurable quantities of either A5 or A9 were found in the fractions from the first (macroglobulin) peak. All fractions belonging to this peak were then pooled and concentrated further (final concentration ~ 100×). In the resulting concentrate small quantities of A5 and A9 were detected as judged by the formation of distinct precipitation lines in the double diffusion test. The concentrations of these allotypic specificities, however, could not be measured by the method of single diffusion. The simultaneous elution of IgG, A5 and A9 (Fig. 2) justifies the conclusion that A9 is associated with IgG. The relation between IgM and A9 must remain uncertain; if it exists it must be confined to a small proportion of the IgM molecules. Alternatively, the presence of small quantities of A9 in the 19*S* peak might arise from 7*S* impurities.

The serum of the A^5/A^9 rabbit was next subjected to immunoelectrophoretic analysis (Fig. 3). The patterns were developed by anti-A9 and anti-A5 sera. (Both these antisera were produced in A^4/A^4 rabbits.) The results of this test confirm the gel-filtration experiment by showing that the molecules which carry A9 specificity have an electrophoretic mobility characteristic of IgG. The precipitation arc developed by anti-A5 serum seems, however, to extend further towards the negative electrode than the precipitation arc developed by anti-A9 serum.

Because of their good immunogenicity, allotypic specificities of the *b* group are widely used in investigations on the structure and synthesis of antibodies. The interaction between two anti-allotype sera often prevented the application of these specificities in certain types of experiments. (Anti-A4 is usually made in A^5/A^5 rabbits and anti-A5 in A^4/A^4 animals; therefore, immunoglobulin present in the first antiserum will react with antibodies present in the second antiserum and vice versa.) If both anti-A4 and anti-A5 antibodies are made in A^6/A^6 rabbits, this interaction can be avoided, but only at the expense of the potency of the resulting antiserum: it can be expected that the stimulation with A5 may be less effective in A^6/A^6 than in A^4/A^4 rabbits, because anti-A5 cross-reacts with A6. In our investigations no evidence of cross-reactivity between A9 and other allotypic specificities was observed. The A^5/A^9 rabbits were found to be as good in the production of anti-A4 as were the A^5/A^5 rabbits. It is therefore likely that A^9/A^9 individuals may be successfully used for the production of both anti-A4 and anti-A5 and that these antisera will not interact. Such non-interacting antisera should be of considerable value in detecting allotypes used as antigenic markers.

Thus we have found a new allotypic specificity which is probably carried by the L-chains of rabbit IgG molecules. The gene controlling this specificity belongs to the *b* locus and thus is allelic to other genes of this group. This hypothesis is based on the following evidence: (1) All heterozygous rabbits in which A9 was detected possessed only one other *b* group specificity (A4 or A5). (2) Rabbits homozygous with respect to A9 do not have any other specificity of the *b* group. (3) Rabbits (Fig. 1, II–5; II–10) in which only A5 and not A4 was detected gave birth to offspring (Fig. 1, III–7,11,12) in which only A4 and not A5 was detected. This would have been impossible if A^9 gene were unrelated to the *b* locus.

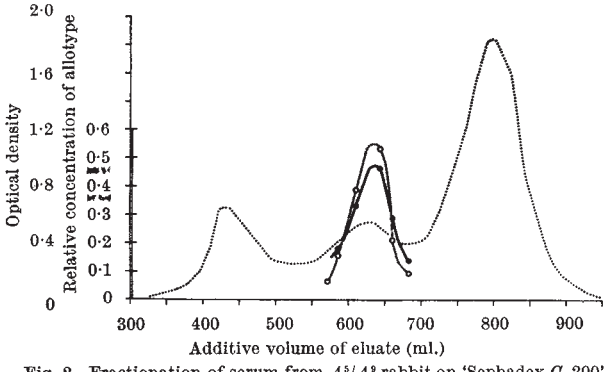

Fig. 2. Fractionation of serum from A^5/A^9 rabbit on 'Sephadex *G*–200'. Optical density (280 mμ) and relative concentrations of A5 and A9 (single diffusion) were plotted against the additive volume of the effluent. . . . . Optical density; O . . . O, relative concentration of A5; ●—●, relative concentration of A9.

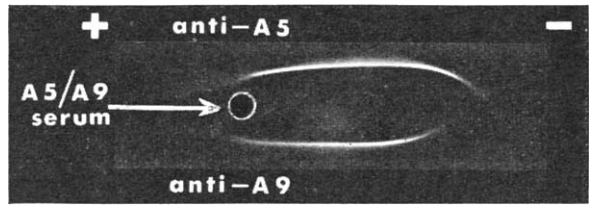

Fig. 3. Immunoelectrophoretic analysis of serum from A^5/A^9 rabbit. The patterns were developed by anti-A5 serum (upper trough) and anti-A9 serum (lower trough). Both antisera were obtained from A^4/A^4 animals. Note the lack of interaction between two antisera.

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1 Dray, S., Dubiski S., Kelus, A., Lennox, E. S., and Oudin, J., *Nature*, **195**, 677 (1962).
2 Oudin, J., *J. Exp. Med.*, **112**, 107 (1960).
3 Oudin, J., *J. Exp. Med.*, **112**, 125 (1960).
4 Dubiski, S., Dudziak, Z., Skalba, D., and Dubiska, A., *Immunology*, **2**, 84 (1959).
5 Dubiski, S., Skalba, D., Dubiska, A., and Kelus, A., *Nature*, **184**, 1811 (1959).
6 Dubiski, S., Dubiska, A., Skalba, D., and Kelus, A., *Immunology*, **4**, 236 (1961).
7 Dray, S., Young, G. O., and Gerald, L., *J. Immunol.*, **91**, 403 (1963).
8 Nisonoff, A., and Thorbecke, G. J., *Ann. Rev, Biochem.*, **33**, 355 (1964).
9 Hamers, R., Hamers-Casterman, C., and Lagnaux, S., *Immunology*, **10**, 399 (1966).
10 Dubiski, S., Rapacz, J., and Dubiska, A., *Acta Genet.*, **12**, 136 (1962).
11 Flodin, P., and Killander, J., *Biochim. Biophys. Acta*, **63**, 403 (1963).
12 Dubiski, S., and Fradette, K., *Proc. Soc. Exp. Biol. and Med.*, **122**, 126 (1966).

Fc Fragment of Immunoglobulin G in Normal Human Plasma and Urine

It has been shown that immunoglobulins of low molecular weight (micro-immunoglobulins) in normal human urine (compare ref. 1) are predominantly composed of material which closely resembles light polypeptide chains[2]. This is also true of the micro-immunoglobulins in "post-exercise" urine[3] and in urine from patients with connective tissue disease[4]. Micro-immunoglobulins in normal human plasma[5–7] include material resembling light chains[2]. Plasma and urine from patients with "heavy chain disease" contain large quantities of protein which is similar to the Fc fragment of IgG, a piece of heavy chains produced by papain digestion[8]. It has been reported that a small part of the micro-immunoglobulins in post-exercise urine[3] and in urine from patients[4] and normal individuals[9] is related to the heavy chain and Fc fragment of IgG. The material related to Fc was not characterized in these reports. Recently, it was demonstrated that protein in normal urine includes material resembling the Fc′ fragment of IgG (ref. 10). Fc′ fragment released by digestion of IgG with papain or trypsin has a smaller molecular size than Fc and is antigenically deficient with respect to this fragment[11].

This communication describes the isolation and identification of a plasma and urinary protein closely resembling the Fc fragment of IgG.

Proteins from normal pooled urine with molecular weights greater than about 10,000 were concentrated 700–1,000 times by ultrafiltration in 'Visking' 23/32 in. dialysis tubing[12]. The concentrates from 24–90 l. of urine and 100–200 ml. samples of fresh plasma from three healthy individuals were subjected to zone electrophoresis on a cellulose column[13] or on 'Pevikon' blocks[12] in 0·1 molar barbital buffer, *p*H 8·6. Fractions corresponding to the β_2 globulin, the fast gamma-globulin and the remaining part of the gamma-globulin regions were pooled and concentrated by ultrafiltration. The urinary β_2 fraction also included the fast gamma-globulin region. The different fractions were separated on 'Sephadex *G*-100' columns equilibrated with 0·1 molar *tris*-hydrochloric acid + 1·0 molar sodium chloride, *p*H 8·0. The distribution in the effluents of components related to IgG, IgA, Fc fragment, Fab fragment, κ chains and λ chains was determined by Ouchterlony gel-diffusion analysis[14] with rabbit antisera against these components.

Fig. 1 shows the results of gel-filtration of the urinary β_2 fraction. The first peak contained protein antigenically related to IgG and IgA. The elution volume of the second peak coincided with that of dimers of free light chains[15]. Material antigenically related to Fc was eluted in the frontal part of this peak. The corresponding peak from the urinary gamma-globulin fraction contained only trace amounts of protein related to Fc.

Gel-filtration patterns of the three electrophoretic plasma fractions revealed small peaks of low molecular weight material (compare ref. 7) which were eluted in a position similar to that of the second peak in Fig. 1. Low molecular weight components were present predominantly in the fast gamma-globulin fraction. All low molecular weight peaks contained protein related to Fc and Fab. Micro-immunoglobulin related to Fc was present mainly in the peaks obtained from β_2 and fast gamma-globulin fractions; it was eluted somewhat earlier than the material related to Fab.

Low molecular weight protein from one fast gamma-globulin fraction crystallized during concentration by ultrafiltration at +4° C. The crystalline material was dissolved in 0·15 molar sodium chloride at room temperature and reacted antigenically similar to Fc. Ultracentrifugal analysis of low molecular weight material from one fast gamma-globulin fraction gave a sedimentation coefficient ($s^0_{20,w}$) of 3·75 *S* in 0·01 molar *tris*-hydrochloric acid + 0·1 molar sodium chloride, *p*H 8·0.

The micro-immunoglobulins related to Fc fragment of IgG present in the β_2 and fast γ fractions of normal human plasma and urine were compared with each other, with the heavy chain and Fc fragment of IgG, and with intact IgG by Ouchterlony gel-diffusion analysis[14], immunoelectrophoresis[16] and urea starch-gel electrophoresis[17]. γ Chains

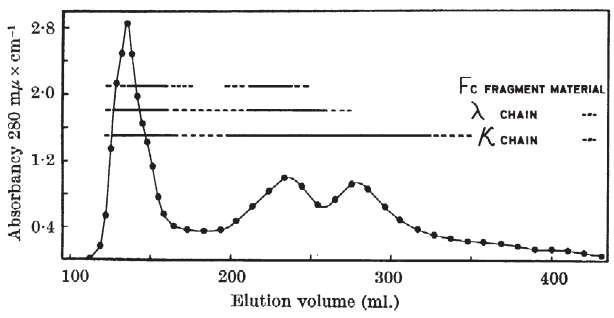

Fig. 1. Gel-filtration on 'Sephadex *G*-100' (2·4 × 117 cm) of the β_2 fraction from normal urine (118 mg protein from 24 l.). The distribution of material reacting with antisera against Fc fragment of IgG and Bence Jones proteins (light chains) of types *K* and *L* are indicated with horizontal lines.

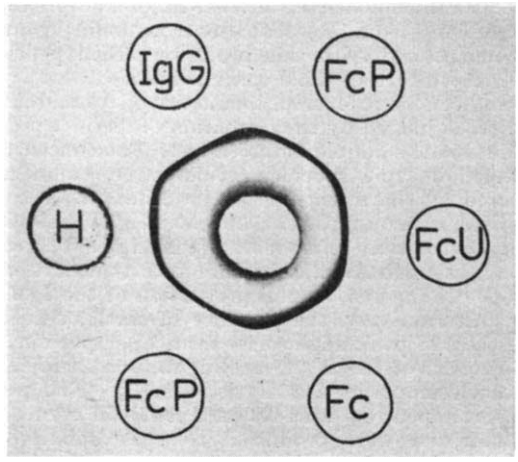

Fig. 2. Double immunodiffusion analysis of micro-immunoglobulins related to Fc fragment of IgG: from normal plasma (*FcP*) and urine (*FcU*); and of IgG, γ polypeptide chains (*H*) and Fc fragment of IgG (*Fc*). The central well contained a rabbit antiserum against γ chains.

were isolated from reduced and alkylated IgG[18]. Fc fragment was isolated from a papain digest of IgG by gel-filtration and DEAE-chromatography on 'Sephadex'[19].

Ouchterlony gel-diffusion analysis using rabbit anti-γ chain serum showed a complete fusion among the precipitin lines given by plasma and urinary micro-immunoglobulins related to Fc, IgG, Fc fragment and γ chains (Fig. 2). Immunoelectrophoretic analyses using rabbit anti-Fc-sera demonstrated that plasma and urine components had similar electrophoretic mobility, which was about the same as that of Fc fragment (Fig. 3).

Low molecular weight components isolated from plasma and urine were examined by starch-gel electrophoresis in urea–formate buffer[17] before and after reduction-alkylation and compared with IgG and Fc fragment treated in a similar way. Reduction and alkylation were carried out at *p*H 8 in the presence of 8 molar urea[17]. All or most of the plasma and urinary proteins treated by reduction-alkylation migrated faster than γ chains but similar to reduced-alkylated Fc fragment of IgG and light chain monomers. In some experiments, small amounts of the reduced-alkylated material seemed to have the mobility of γ chains.

The starch-gel electrophoresis experiments suggested that at least part of the plasma and urinary proteins which were antigenically related to Fc could be split by reduction in the same way as Fc fragment[1]. Further evidence was obtained by gel-filtration in 0·5 molar propionic acid on 'Sephadex *G*-100' of a reduced and alkylated low molecular weight fraction isolated from plasma. This reduction was carried out in the absence of urea to obtain soluble products[17]. Two protein peaks were obtained. Both contained material antigenically related to Fc as revealed by Ouchterlony gel diffusion analysis with rabbit anti-Fc-sera. The second peak was eluted somewhat earlier than that of light chain monomers.

The results of the present investigation indicate that β_2 and fast γ fractions of normal human plasma and urine contain small quantities of low molecular weight components which closely resemble Fc fragment of IgG in their antigenic and electrophoretic characteristics, in molecular size and in their properties after reduction. The fact that part of the protein related to Fc from one sample of plasma crystallized gives additional support for a very close relationship to Fc fragment.

The "Fc fragment" present in normal human plasma and urine may arise from *de novo* synthesis, as seems to be true for its possible pathological counterpart the "heavy-chain disease" protein[20] and for normally occurring free light chains[9,21]. Alternatively, it may derive from enzym-

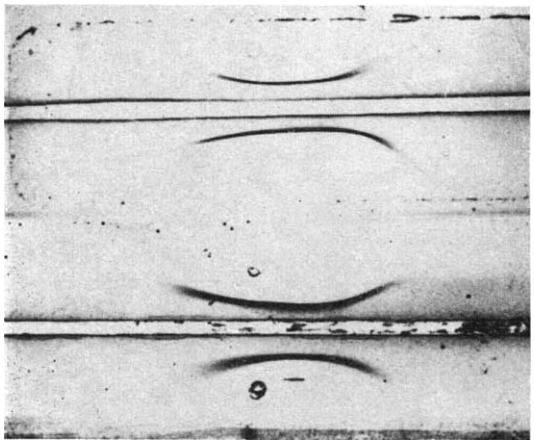

Fig. 3. Immunoelectrophoretic comparison of micro-immunoglobulins from β_2-fractions of normal plasma (*FcP*) and urine (*FcU*) with Fc fragment of IgG (*Fc*), using a rabbit anti-Fc-fragment serum. The anode is to the left.

atic degradation of IgG or from both *de novo* synthesis and enzymatic degradation. Several investigators have observed degradation of IgG on storage in solution (compare ref. 22). Components with Fc and Fab characteristics have been isolated from stored IgG (ref. 22). These components closely resembled fragments from IgG treated with plasmin. It was suggested that the degradation occasionally observed during storage may result from the presence of plasmin in the preparations. An investigation reported in a recent abstract[9] seems to indicate that micro-immunoglobulins related to the Fc fragment of IgG in normal human urine derive from catabolism of IgG. The material described therein, however, was not adequately characterized as regards its resemblance to Fc and Fc′ fragments. The study of Turner and Rowe[10] has shown that normal urine contains protein similar to the Fc′ fragment of IgG, whereas the present investigation demonstrates the normal occurrence of urinary and plasma protein closely resembling the Fc fragment of IgG.

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[1] Cohen, S., and Porter, R. R., in *Adv. Immunol.*, **4**, 287 (Academic Press, New York, 1964).
[2] Berggård, I., and Edelman, G. M., *Proc. U.S. Nat. Acad. Sci.*, **49**, 330 (1963).
[3] Freedman, M. H., and Connell, G. E., *Canad. J. Biochem.*, **42**, 1065 (1964).
[4] Hanson, L. Å., and Tan, E. M., *J. Clin. Invest.*, **44**, 703 (1965).
[5] Berggård, I., *Clin. Chim. Acta*, **6**, 545 (1961).
[6] Takahashi, S., and Schmid, K., *Biochim. Biophys. Acta*, **63**, 343 (1962).
[7] Porath, J., and Ui, N., *Biochim. Biophys. Acta*, **90**, 324 (1964).
[8] Franklin, E. C., *J. Exp. Med.*, **120**, 691 (1964).
[9] Vaughan, J. H., Jacox, R. F., and Gray, B., *Fed. Proc.*, **25**, 374 (1966).
[10] Turner, M. W., and Rowe, D. S., *Nature*, **210**, 130 (1966).
[11] Poulik, M. D., *Nature*, **210**, 133 (1966).
[12] Berggård, I., *Arkiv Kemi*, **18**, 291 (1962).
[13] Porath, J., *Science Tools*, **11**, 21 (1964).
[14] Ouchterlony, Ö., in *Prog. in Allergy*, **5**, 1 (Karger, S., Basel, 1958).
[15] Berggård, I. (in preparation).
[16] Scheidegger, J. J., *Intern. Arch. Allergy App. Immunol.*, **7**, 103 (1955).
[17] Edelman, G. M., and Poulik, M. D., *J. Exp. Med.*, **113**, 861 (1961).
[18] Fleischman, J. B., Pain, R. H., and Porter, R. R., *Arch. Biochem. Biophys.*, Suppl. 1, 174 (1962).
[19] Bennich, H. (in preparation).
[20] Franklin, E. C., Lowenstein, J., Bigelow, B., and Meltzer, M., *Amer. J. Med.*, **37**, 332 (1964).
[21] Stevenson, G. T., *J. Clin. Invest.*, **41**, 1190 (1962).
[22] Connell, G. E., and Painter, R. H., *Canad. J. Biochem.*, **44**, 371 (1966).

Effect of Endotoxin on Resistance in the Early Period of Ontogenesis

ORGANISMS react to bacterial antigens in the early stages of ontogenesis, and it has been reported that bacterial antigens raise the resistance of the embryo[1–3], while the antigenic stimulation of the embryo[4] and the subsequent second vaccination of newly hatched chicks considerably enhance the resistance of the chicks in their first few weeks of life[5]. Our previous studies have shown that it is best to perform the first stimulation on the twelfth day of incubation, and the second vaccination on the first day after hatching[6].

Table 1. EFFECT OF ENDOTOXINS AND OF BACTERIAL ANTIGENS

Experiment	Preparation*	Dose†	Number of infected chicks	Died within 21 days	Percentage‡ of chicks which died
I	Bacterial antigen *S. gallinarum* 15-*S*	$1{\cdot}2\times10^8$	35	19	54·3 ($P<0{\cdot}05$)
	Endotoxin *S. gallinarum* 15-*S*	310 μg N 510 μg P	44	23	52·3 ($P<0{\cdot}01$)
	Control group		31	26	83·9
II	Endotoxin *S. gallinarum* 15-*S*	375 μg N 594 μg P	92	29	31·5 ($P<0{\cdot}001$)
	Endotoxin *E. coli* 0111*B*4 : *H*2	200 μg N 361 μg P	105	51	48·6 ($P<0{\cdot}05$)
	Endotoxin *E. coli* 11*ME*5	192 μg N 429 μg P	84	32	38·1 ($P<0{\cdot}001$)
	Control group		87	59	67·8
III	Bacterial antigen *S. gallinarum* 15-*S*	6×10^7	165	131	79·4 ($P<0{\cdot}001$)
	Endotoxin *S. gallinarum* 15-*S*	375 μg N 594 μg P	94	73	77·7 ($P<0{\cdot}001$)
	Endotoxin *E. coli* 0111*B*4 : *H*2	200 μg N 361 μg P	40	32	80·0 ($P<0{\cdot}05$)
	Endotoxin *E. coli* 11*ME*5	192 μg N 429 μg P	101	79	78·2 ($P<0{\cdot}001$)
	Control group		156	145	92·9
IV	Bacterial antigen *S. gallinarum* 15-*S*	4×10^7	172	120	69·8 ($P<0{\cdot}001$)
	Bacterial antigen *E. coli* 0111*B*4 : *H*2	4×10^7	76	51	67·1 ($P<0{\cdot}001$)
	Bacterial antigen *E. coli* 11*ME*5	4×10^7	73	55	75·3 ($P<0{\cdot}001$)
	Control group		117	113	96·6

* Formalin killed bacterial suspensions (*E. coli* or *S. gallinarum*) in 0·9 per cent sodium chloride solution or the phenol isolated endotoxin in saline were used as a preparation.

† The dosage of vaccine used to inoculate the embryo is expressed by the number of injected bacteria/0·1 ml. of vaccine; for the endotoxin by nitrogen (N) and phosphorus (P) content in μg/0·1 ml. of the preparation. The newly hatched chicks were inoculated by a double dose (0·2 ml.) of the same antigen.

‡ The effect of the preparations was ascertained by the χ^2 method, comparing vaccinated chicks with the unvaccinated controls.

The object of the present investigation was to determine whether endotoxin exercises a stimulating effect on chick resistance in the early stages of ontogenesis similar to that of bacterial antigen. The effects of the endotoxins isolated by phenol[7] from both a smooth strain (0111 : *B*4 : *H*2) and a rough strain (11 *ME* 5) of *Escherichia coli* as well as from a smooth strain of *Salmonella gallinarum* (15-*S*) were compared. At the first inoculation 0·1 ml. of endotoxin was injected into the egg air-chamber on the twelfth day of incubation. At the second inoculation 0·2 ml. of endotoxin was injected on the first day after hatching out. On the day after revaccination, the chicks were infected with a live culture of *S. gallinarum* by injecting 0·2 ml. of it ($1{\cdot}2\times10^8$ bacterial cells) subcutaneously into the thigh region. Experiments were carried out on White Leghorns. Chick mortality was observed over a period of 21 days (see Table 1). It appears that all the preparations exercised a stimulating effect on the resistance of the chicks. On comparing the effect of the endotoxins of the rough and the smooth strain of *E. coli*, no statistically significant difference in the action of these preparations was found (in experiment II, 38·1 and 48·6 per cent, $\chi^2=1{\cdot}88$, $P>0{\cdot}05$). Also there was no difference statistically between the effect of the endotoxins of *E. coli* and *S. gallinarum* (in experiment II, 31·5 and 48·6 per cent, $\chi^2=2{\cdot}35$, $P>0{\cdot}05$).

It is clear that in the first 3 weeks of the life of a chick a noticeable increase in resistance to infection is brought about by preparations of *S. gallinarum* and *E. coli*.