lar compartment, originally decreased, showed a delayed rehydration to normal levels. Hence the voluntary water intake showed more positive correlation with changes in intracellular volume than with extracellular volume change. Fluid balance was affected both by intracellular hydration and electrolyte levels in the extracellular fluid.

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Widened Reactivity of Antibody Produced by Prolonged Immunization.

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A number of workers have observed that antibody becomes more cross-reactive as the course of immunization is prolonged. The first observations were apparently those of Magnus[1] on antigenic plant-extracts. Many similar results have been obtained with antigenic cells or crude mixtures of antigens; the first evidence that reasonably pure proteins can produce the same effect was that of Wells and Osborne.[2] We used 5-times recrystallized ovalbumins from hen and duck and offered additional proof[3] that the antibodies to a single "pure" antigen may develop a broadened reactivity as immunization proceeds. The antisera from later bleedings *may* show a broader equivalence-zone,[4] and the antibody, in the zone of antigen-excess, can combine with a larger quantity of antigen.[4, 5]

Three possible explanations of these effects have been considered; conceivably all of them might apply in some instances.

1. The antibody initially formed is directed toward a dominant determinant-group of the antigen, and, progressively, additional antibodies are formed for separate, minor determinants.[6-9]

2. The later antibodies differ from the earlier by the presence on

1 Magnus, *Ber. deut. Bot. Gesellsch.*, 1908, **26a**, 532 (cited in[2]).
2 Wells, H. G., and Osborne, T. B., *J. Inf. Dis.*, 1913, **12**, 341; 1916, **19**, 183.
3 Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1934, **26**, 469; 1936, **30**, 41.
4 Heidelberger, M., and Kendall, F. C., *J. Exp. Med.*, 1935, **62**, 697.
5 Malkiel, S., and Boyd, W. C., *J. Exp. Med.*, 1937, **66**, 383.
6 Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1933, **25**, 61.
7 Heidelberger, M., and Kendall, F. C., *J. Exp. Med.*, 1934, **59**, 519.
8 Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1938, **67**, 709.
9 Pauling, L., *J. Am. Chem. Soc.*, 1940, **62**, 2643.

their molecules of additional discrete reactive groups that may differ qualitatively, the later antibodies thus being more multivalent.[4, 10, 11]

3. The later antisera contain antibodies whose combining groups, although not necessarily more than one per molecule, possess a wider affinity, which permits combination with a larger number of related chemical groups, perhaps because the reactive patch on the molecule of antibody is larger and its pattern is more complex. Combination with related heterologous haptens or antigens might be effected by limited portions of the chemically active groups constituting the pattern of the antideterminant, the whole structure being brought into play only in the presence of the complete (homologous) determinant.

The third possibility is substantially an extension of the explanation offered by Landsteiner[12] to account for the multiplicity of antibodies formed to one determinant-group (metanilic acid); that is, the antibodies " . . . . vary to some extent around a main pattern." Such structural similarities also seem adequate to explain the cross-reactivity of antisera to various pure ovalbumins.[13] The variation among antibodies is strikingly shown by absorption with heterologous antigens and we think that the behavior of such absorbed sera, when further treated with heterologous hapten[14] or antigen,[13] offers support for the above-mentioned concept that cross-reactions may often depend on the activity of only a portion of the pattern of chemical groups (perhaps not necessarily even adjacent groups) composing the reactive patch on the molecule of antibody.

We may point out that the antibodies to hen-ovalbumin[13] (H) could be separated by absorption with turkey-ovalbumin (T) into at least two kinds: those able to precipitate T and those which could not. The latter were nevertheless prevented from precipitating H, by the addition of a 16- to 32-fold relative excess of T. The indicated conclusion is that all of the anti-hen precipitins had the power of reacting with T, but that those which could precipitate it—and according to our own experience these would be produced later in the course of immunization—had a still coarser specificity, enabling them to combine more firmly and produce an insoluble compound.

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10 Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1940, **71**, 263.

11 Heidelberger, M., Treffers, H. P., and Mayer, M., *J. Exp. Med.*, 1940, **71**, 271.

12 Landsteiner, K., *The Specificity of Serological Reactions*, C. C. Thomas, Baltimore, 1936, p. 143.

13 Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1940, **71**, 445.

14 Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1936, **63**, 325; 1931, **54**, 295.

During a study[15] of the cross-reactivity of antisera to *p*-amino-benzoic-acid-azohemocyanin *(Limulus)* we tested the inhibitive capacity of a number of uncoupled compounds more or less related to the prosthetic group of this compound antigen, and observed that some antisera from later bleedings contained antibody capable of being inhibited by cyclohexane carboxylic acid, whereas this compound did not inhibit precipitation by antibody from earlier bleedings of the same animals. We were skeptical of the accuracy of the first observation but have repeatedly confirmed it.

Method. After preliminary titrations to determine the optimal amounts of immune serum and test-antigen a quantity of the latter, *p*-amino-benzoic-acid-azocasein, sufficient to give a concentration of 2 γ N/ml was mixed with 5% gelatin in salt solution.[16] About 0.2 ml amounts of this mixture were distributed in small tubes (5 mm bore) and chilled. Equal parts of diluted serum and the serially diluted compound being tested for inhibitive property were mixed and overlayered on the preparation of antigen, and the tubes were kept at 37° for 5 hours. Reactions at the sharp interface thus ensured were recorded at hourly intervals. Inhibition by very small amounts of some compounds was complete even after refrigeration overnight. The compounds were adjusted to pH 7.4 or as near thereto as solubility permitted. They did not inhibit unrelated sera.

An example of the inhibitive behavior of cyclohexane carboxylic acid (CCA) is given in Table I. Similar results were obtained with sera from another rabbit but not from a third. It will be noted that the disparity between the effectively inhibitive amounts of the different haptens is much greater in the case of the 2nd than of the 3rd or 5th bleeding. The fact that the early antibody is inhibited by the homologous hapten and not by CCA whereas later antibody

TABLE I.

Precipitation of Casein-azo-*p*-aminobenzoic Acid with Antihemocyanin-azo-*p*-aminobenzoic Acid Inhibited by Simple Haptens.

	p amino benzoic acid			Benzoic acid			Cyclohexane carboxylic acid		
	Micromols required to inhibit completely for								
Serum*	1	2	3	1	2	3	1	2	3 hr
562_2 1:10	.174	.174	.260	.392	.588	.588	not by 2.00		
562_3 1:6	.077	.116	.174	.077	.174	.260	.116	.392	.588
562_5 1:4	.077	.174	.260	†	†	†	.116	.174	.260

*Subscripts indicate number of bleeding of rabbit 562.
†Not tested.

[15] Hooker, S. B., and Boyd, W. C., *Arch. Path.*, 1939, **28**, 754.

[16] Hanks, J. H., *J. Immunol.*, 1935, **28**, 95.

is temporarily, but completely, inhibited by CCA indicates that the early kind of antibody has virtually disappeared from the serum and has been replaced by antibody capable of combining with the heterologous hapten. In this instance an interval of 7 weeks had elapsed between the 2d and 3d bleedings—an interval probably far longer than necessary for the elimination of early antibody and for a fresh antibody-response to the injections of antigen given during this intervening period.

The development of this broadened reactivity seems to be accounted for best by the third explanation offered above. The mechanism suggested in the first, which is surely sometimes operative, is less applicable in this case which concerns a relatively simple hapten; the second possibility, involving an increased valence (in the sense of discrete combining patterns) of the antibody-molecule, can hardly apply at all.

Structurally, the haptens here considered are grossly very similar and the influence of finer configurational differences among them must be invoked to explain the results obtained. One may consider the influence of the presence of double bonds[17] and an (altered) NH_2 group which might dominate the production of early antibody; different angles between the carbon atoms in the two kinds of ring, or different planarities of the rings as wholes. We are informed that the oxygen atoms in benzoic acid would tend to form stronger hydrogen bonds with proton-donating groups than would those in CCA; if hydrogen bonds are concerned in the union of antigen with antibody[18] then their influence on specificity would appear to be greater in the case of the early antibody, which was inhibited by benzoic acid but not by CCA. Whether any of these possible influences is operative cannot be decided at present; the nature of the altered mechanism of antibody-formation is entirely obscure.

Summary. Specific precipitation by antibody produced early in the course of immunization with *p*-amino-benzoic-acid-azohemocyanin was not inhibited by a related heterologous hapten, cyclohexane carboxylic acid. As immunization continued the antibodies were replaced by one that was strongly inhibited by this hapten. Some possible reasons for this broadened reactivity are discussed.

[17] Erlenmeyer, H., and Berger, E., *Arch. exp. Path. u. Pharm.*, 1934, **177**, 116.

[18] Pauling, L., *The Nature of the Chemical Bond*, Cornell Univ. Press, Ithaca, 1939, p. 411.