[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND BACTERIOLOGY, DUKE UNIVERSITY SCHOOL OF MEDICINE]

A Note on the Effect of Choline Chloride on the Kahn Flocculation Reaction[1]

BY ELLIOT VOLKIN,[2] JOHN O. ERICKSON, H. W. CRAIG AND HANS NEURATH

The extent of flocculation of antibodies by antigens is known to depend on external conditions such as *p*H, electrolyte concentration and temperature.[3] Maximal flocculation requires an optimal range of each factor and flocculation may be inhibited outside this range. This type of inhibition is *non-specific* and may be produced in varying degrees in almost any serological system. In contrast, inhibition by haptens is *specific* and is believed to result from a competition between the antigen and the antigenically related hapten for the reactive groupings on the antibody.[4] The latter reaction requires but minute quantities of the hapten.

Extension of hapten inhibition studies to flocculating systems involving lipoidal antigens is impeded by the ill-defined chemical composition of the antigen, particularly the lipoidal beef heart antigen preparations commonly used in the serological diagnosis of syphilis.[5,6] While inhibition has been reported in several instances, it has been brought about by variation of external conditions rather than by the presence of specific haptens.[7] Recently, however, experiments were described purporting to demonstrate a specific inhibitory effect of choline chloride, β-ethanolamine hydrochloride and of a lipoidal antigenic extract on the reaction between syphilitic sera and Kahn antigen.[8] This effect was postulated to be due to hapten inhibition and was interpreted in terms of a general theory.[9] During the past year, two inhibition reactions in the serological titration with the Kahn and other lipoidal antigens have been described. They are due to (1) a heat labile[10,11] and (2) a heat stable[12,13] component of human serum and are being further studied with respect to the serological differentiation between truly syphilitic and biologic false positive reactions. Newburgh's results appear of particular interest in this connection and have prompted us to repeat his experiments under more rigorously controlled conditions.

Examination of the experimental data reveals that relatively high concentrations of choline chloride and β-ethanolamine hydrochloride are required for marked inhibition. Indeed, the lowest effective concentration was of the order of 0.7 *M*, whereas the highest value was about 3.6 *M*. Such concentrations are out of proportion to those usually required for inhibition by haptens (10^{-7}–$10^{-9}M$).[9] Further, since both choline chloride and β-ethanolamine hydrochloride are strong electrolytes, the question arises to what extent the observed effects are solely due to the increase in electrolyte concentration. In order to eliminate the influence of ionic strength, it is necessary to compare the effect of these organic salts with that obtained by equimolar concentrations of antigenically inert ions of like valence, *i. e.*, sodium chloride. The results of such experiments are presented below.

Experimental

Individual serum specimens of varying titer were obtained from patients known to have syphilis. With one exception (no. 931), no treatment was administered prior to the time the blood was drawn. The quantitative Kahn flocculation test was done in the usual manner, the amounts of antisera and antigen being as given in the tables. Prior to analysis, the sera were heat-inactivated at 62° for three minutes. The degree of flocculation was estimated from the microscopic appearance of the floccules, except for the macroscopic readings required for experiments in which the salts were added after formation of the floccules. In the tables, the size of the floccules, representing the intensity of the reaction, is indicated by numerical figures, *i. e.*, 4, 3, 2, 1, ± and 0 (negative).

Stock solutions of 6 *M* choline chloride and 6 *M* sodium chloride were prepared and dilutions made therefrom. The final molarities of the salts approximated those of Newburgh's experiments[8] while in supplementary experiments they were extended also to lower ranges.

Results[14]

1. Effects of Choline Chloride and Sodium Chloride for Varying Antigen/Antibody Ratios.—As shown in Table I, the amount of antigen was varied two-fold from 0.005 to 0.08 cc., the concentration of the electrolytes in the mixtures being 3.6 *M*. The control experiment (line 1) contained the standard saline–phosphate buffer (0.15 *M* sodium chloride, 0.02 *M* phosphate, *p*H 6.8). It may be seen that inhibition by 3.6 *M* sodium chloride was of the same order as that by

(1) This work was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Duke University.

(2) Abbott Laboratories Research Fellow in Biochemistry.

(3) *Cf.* W. C. Boyd, "Fundamentals of Immunology," Interscience Publishers, Inc., New York, 1943, Chapter VI.

(4) W. C. Boyd, *ibid.*, Chapter II.

(5) H. Eagle, "Laboratory Diagnosis of Syphilis," C. V. Mosby Co., St. Louis, 1937, chap. III.

(6) For a chemically better defined antigen, see M. C. Pangborn, *J. Biol. Chem.*, **143**, 247 (1942).

(7) *Cf.* R. L. Kahn, *Univ. Hosp. Bull.* (Ann Arbor, Michigan), **8**, 45 (1942).

(8) J. D. Newburgh, THIS JOURNAL, **67**, 779 (1945).

(9) D. Pressman, J. T. Maynard, A. L. Grossberg and L. Pauling, *ibid.*, **65**, 728 (1943).

(10) C. R. Rein and L. Pillemer, quoted in C. R. Rein and E. S. Elsberg, *Am. Jour. Clin. Path.*, **14**, 461 (1944).

(11) *Cf.* E. E. Ecker, G. L. Castro and S. Seifter, *Proc. Soc. Exp. Biol. Med.*, **58**, 95 (1945).

(12) H. Neurath, *et al.*, *Science*, **101**, 68 (1945).

(13) H. Neurath, *Venereal Disease Inform.*, Suppl. No. 20 (1945) (in press).

(14) For reasons of economy of space, only representative data are given below for each group of experiments. Additional data are on file in this Laboratory.

choline chloride. Here, as in all other experiments, neither of the two salts caused flocculation of the antigen in the absence of serum. Though the effects produced by lower concentrations of these salts (0.25 to 0.06 *M*) were of smaller magnitude, nevertheless, sodium chloride was found to be at least as active in causing partial inhibition as was choline chloride.

TABLE I

EFFECTS OF CHOLINE CHLORIDE AND SODIUM CHLORIDE AT VARIOUS RATIOS OF ANTIGEN AND ANTIBODY

0.6 cc. of diluent added to 0.4 cc. of serum. Tests made on 0.1 cc. of diluted serum.

Serum No.	Diluent	Concn. of diluent *M*[a]	Antigen suspension, cc. × 1000 5	10	20	40	80
1058	Saline buffer	—	4	4	2	±	0
1058	Choline chloride	6	0	0	0	0	0
1058	Sodium chloride	6	±	±	0	0	0
1092	Saline buffer	—	4	4	3	2	1
1092	Choline chloride	6	0	0	0	0	0
1092	Sodium chloride	6	1	±	0	0	0
1221	Saline buffer	—	4	4	3	2	1
1221	Choline chloride	4	1	±	0	0	0
1221	Potassium chloride	4	4	3	1	0	0
1221	Sodium chloride	4	3	1	0	0	0
1221	Lithium chloride	4	0	0	0	0	0

[a] Except for saline buffer.

TABLE II

EFFECT OF EQUIMOLAR CONCENTRATIONS OF CHOLINE CHLORIDE AND SODIUM CHLORIDE IN THE REGION OF ANTIGEN EXCESS

Antigen suspension, 0.025 cc.

Serum no.	Diluent 0.75 *M*[a]	Serum dilution 1	1/2	1/4[b]
1058	Saline buffer	3	2	0
1058	Choline chloride	0	0	0
1058	Sodium chloride	±	±	0
1130	Saline buffer	3	2	1
1130	Choline chloride	±	0	0
1130	Sodium chloride	1	1	±
1117	Saline buffer	4	2	1
1117	Choline chloride	0	0	0
1117	Potassium chloride	1	0	0
1117	Sodium chloride	1	0	0
1117	Lithium chloride	±	0	0

[a] Except for saline buffer. [b] At higher dilutions the readings were zero in all cases.

2. Effects in the Region of Antigen Excess.—In these experiments sera were titrated in serial two-fold dilutions, using 0.025 cc. of antigen throughout. In concentrations of 0.75 *M*, choline chloride was slightly more effective in producing inhibition than was sodium chloride. Lower concentrations of these salts, *i. e.*, 0.25 and 0.06 *M* were without effect.

The effects of both salts on the redispersion of floccules formed in the region of antigen excess are given in Table III. While redispersion by choline chloride was slightly faster than by sodium chloride, after the third addition redispersion was practically complete in either case. It is to be noted that mere addition of buffer by itself caused considerable redispersion of the floccules.

TABLE III

EFFECT OF ADDITION OF CHOLINE CHLORIDE AND SODIUM CHLORIDE AFTER FORMATION OF PRECIPITATE IN THE REGION OF ANTIGEN EXCESS

Antigen suspension, 0.025 cc. Concentration of the diluents other than saline buffer was 6 *M*.

Serum no.	Reading after 0.1 cc. diluent addition of	Serum dilution 1	1/2	1/4[a]
1058	None	4	4	2
1058	1st Saline buffer	4	2	0
1058	2nd Saline buffer	4	2	0
1058	3rd Saline buffer	4	2	0
1058	1st Choline chloride	±	0	0
1058	2nd Choline chloride	0	0	0
1058	3rd Choline chloride	0	0	0
1058	1st Sodium chloride	2	1	±
1058	2nd Sodium chloride	±	0	0
1058	3rd Sodium chloride	0	0	0
1117	None	4	2	1
1117	1st Saline buffer	4	2	±
1117	2nd Saline buffer	4	2	±
1117	3rd Saline buffer	4	2	0
1117	1st Choline chloride	1	±	0
1117	2nd Choline chloride	±	0	0
1117	3rd Choline chloride	0	0	0
1117	1st Sodium chloride	4	2	±
1117	2nd Sodium chloride	2	1	±
1117	3rd Sodium chloride	0	0	0

[a] At higher dilutions the readings were zero in all cases.

3. Effects in the Region of Antibody Excess.—Though the extent of flocculation is usually unaffected by excess of antibody, certain syphilitic sera may show inhibition when tested in low dilutions. While the nature of these zone phenomena is unexplained,[15] the irregularity of occurrence renders this inhibition unlikely to be due solely to antibody excess. Accordingly, the stated influence of choline chloride in abolishing these zones[8] cannot be readily ascribed to a hapten effect on antibody/antigen ratios.

In Table IV the effects of equimolar concentrations of choline chloride and sodium chloride on the serological titrations of selected high-titer syphilitic sera with varying amounts of antigen are presented.

The results are at variance with those of Newburgh[8] in that choline chloride exerted an inhibiting influence rather than causing additional flocculation, regardless of the amount of antigen present. In contrast, sodium chloride partly eliminated the zones in low antigen concentrations and produced partial inhibition in the presence of larger amounts of antigen.

When choline or sodium chloride were added subsequent to the formation of the floccules, both electrolytes were found to cause additional flocculation, a slightly stronger effect being observed

(15) Ref. 4, chapter X.

TABLE IV

EFFECT OF EQUIMOLAR CONCENTRATIONS OF CHOLINE CHLORIDE AND SODIUM CHLORIDE IN THE REGION OF ANTIBODY EXCESS

0.5 cc. of diluent added to 0.5 cc. of serum. Tests made on 0.1 cc. of diluted serum. Concentration of the diluents other than saline buffer was 6 *M*.

Serum no.	Diluent	Antigen suspension, cc. × 1000 5	10	20	40	80
931	Saline buffer	1	3	4	4	2
931	Choline chloride	1	1	±	0	0
931	Sodium chloride	2	3	2	1	±
992	Saline buffer	1	3	4	4	2
992	Choline chloride	1	1	±	0	0
992	Sodium chloride	3	2	1	±	±
263	Saline buffer	2	4	4	3	2
263	Choline chloride	±	±	0	0	0
263	Sodium chloride	2	4	3	2	1

TABLE V

EFFECT OF ADDITION OF CHOLINE CHLORIDE AND SODIUM CHLORIDE AFTER FORMATION OF PRECIPITATE IN THE REGION OF ANTIBODY EXCESS

Tests made on 0.15 cc. of diluted serum. Concentration of the diluents other than saline buffer was 6 *M*.

Serum no.	Reading after 0.1 cc. diluent addition of	Antigen suspension, cc. × 1000 5	10	15	20	40	80
931	None	2	2	2	3	4	4
931	1st Saline buffer	2	2	2	3	4	4
931	2nd Saline buffer	2	2	2	3	4	4
931	3rd Saline buffer	2	2	2	3	4	4
931	1st Choline chloride	3	4	4	4	4	4
931	2nd Choline chloride	4	4	4	4	4	4
931	3rd Choline chloride	4	4	4	4	4	4
931	1st Sodium chloride	2	3	3	4	4	4
931	2nd Sodium chloride	2	4	4	4	4	4
931	3rd Sodium chloride	2	4	4	4	4	4
564	None	2	2	2	2	4	4
564	1st Saline buffer	2	2	3	3	4	4
564	2nd Saline buffer	2	2	3	3	4	4
564	3rd Saline buffer	2	2	3	3	4	4
564	1st Choline chloride	2	3	4	4	4	4
564	2nd Choline chloride	2	4	4	4	4	4
564	3rd Choline chloride	2	4	4	4	4	4
564	1st Sodium chloride	2	2	3	4	4	4
564	2nd Sodium chloride	2	3	4	4	4	4
564	3rd Sodium chloride	2	3	4	4	4	4

for choline chloride than for sodium chloride. Control experiments with the saline-phosphate buffer failed to show any effect of additional shaking (Table V).

4. Effects of Other Cations.—Though the general effects described for choline chloride have been found to be producible also by equimolar concentrations of sodium chloride, the former salt appears to be somewhat more effective than the latter (Tables I, II, III and V). These differences may well be due to the different positions of the corresponding cations in the lyotropic series. Accordingly, the experiments described in Tables I and II for choline and sodium chloride were repeated using also equimolar concentrations of lithium and potassium chloride, the peptizing effect of the lithium ion being generally greater, and that of the potassium ion less, than that of the sodium ion. The results are given in the lower parts of Tables I and II. The data indicate lithium chloride to be as effective as choline chloride, whereas potassium chloride is less effective than any of the other salts studied.

Summary

The data presented in this paper demonstrate that the effects observed for choline chloride[8] on the serological reaction with the Kahn antigen may likewise be produced by equimolar concentrations of sodium chloride. It is concluded that this type of inhibition is *non-specific*, resulting merely from a peptizing effect produced by high concentrations of these and other uni-univalent electrolytes rather than from a specific hapten effect. In addition, a denaturing effect of relatively high concentrations of choline chloride on the antibody protein, similar to that produced by guanidine hydrochloride,[16] has to be considered in explanation of the present observations. These conclusions do not necessarily apply to the inhibitory effect of the Kahn antigenic extract,[8] the mechanism of which remains to be further elucidated.

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(16) *Cf.* J. O. Erickson and H. Neurath, *J. Gen Physiol.*, **28**, 421 (1945).