aerosol a longer time before the symptoms of asthma occurred. It has been calculated by the method of normal variables that a significant difference exists between the means of the times of exposure of the two groups. A value of $t = 5.5$ was obtained which indicates a significant difference with a confidence limit of better than 98%.

After a further 2 week rest period the control group of guinea pigs became the experimental, hormone-treated group, and vice versa. A significant difference, $t = 4.7$, was calculated for the means of these two groups.

The amounts of antiserum and the concentration of the antigen used were determined experimentally so that the appearance of asthma would neither occur too rapidly nor too slowly in the control group. It was found that the quantity of cortisone used and the time interval allowed to elapse before challenge were important criteria. The animals showed individual variation as to the rapidity with which asthma was produced, effectiveness of hormonal treatment, etc. This variation was not necessarily consistent in a repeated trial. None of the untreated, control guinea pigs failed to show typical symptoms of asthma, however, within the ten minute time limit which was arbitrarily chosen. Most of the cortisone-treated animals which survived a 10 minute exposure failed to show any of the more typical symptoms of asthma after they were out of the chamber; an increased respiratory rate was noticed in some of these animals.

Cortisone has been reported as being effective in suppressing experimental hypersensitivity of the Arthus type(3), in inhibiting the cardiovascular and renal lesions of hypersensitivity in rabbits(4), in preventing the anaphylactoid reaction in rats(5), in protecting mice (6) and rats(7) against anaphylaxis and guinea pigs against reversed passive anaphylaxis(8). The hormone has been found to be ineffective in specific antibody-antigen reactions resulting in anaphylaxis by the usual active and passive technics. The results herein reported indicate that cortisone does play a protective role in the anaphylactic type of hypersensitivity. Preliminary results indicate that ACTH also exerts a somewhat similar effect.

Summary. In a significant percentage of sensitized guinea pigs cortisone was found to be effective in inhibiting or delaying the symptoms of asthma induced by an aerosolized antigen.

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Determination of Quaternary Ammonium Compounds Including Acetylcholine, Tetraethylammonium, and Hexamethonium.*† (19791)

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The increasing pharmacological interest in quaternary ammonium compounds (QAC) stimulated our efforts to develop a method for their determination in biological media. The methyl orange method of Brodie(1,2) and the bromcresol purple method of Woods(3) are general reactions for organic bases, and

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therefore, limited in their usefulness because of formation of the dye complex with normally occurring organic bases extractable from the biological material. The major problem in finding a suitable method centered around the difficulty of quantitatively extracting the highly ionized QAC from biological media without contamination. The industrial procedures, which have been summarized by Dubois(4,5), and the precipitation methods, such as the reineckate salt method, lack the required sensitivity. The bromphenol blue method of Auerbach(6), however, seemed of greatest promise, and the method to be reported here is a modification of this. The great advantage of this bromphenol blue method is that it circumvents the necessity of first extracting the QAC from the test media. The reaction between the anionic bromphenol blue and the quaternary cation can be carried out, in the presence of alkali, in the original biological media and then the dye complex that is formed can be quantitatively extracted without interference from the unreacted dye or from any of the naturally occurring organic bases. The procedure as outlined below increased the sensitivity of Auerbach's method (6) more than tenfold. Smaller quantities of sample are used, but the increase in sensitivity is due mainly to the addition of phosphate-ion which was found to be essential for good color development. This reaction to form a complex salt in alkaline solution seems to be a property only of QAC and not of tertiary nitrogen compounds. Auerbach(6) tested 50 tertiary amines with negative results. Using the method reported here, negative results were also obtained with a number of the tertiary amines he tested as well as with other tertiary nitrogen compounds such as procaine.

The method to be reported has been divided into two main sections: 1) a general procedure and 2) a modified procedure.

General procedure. To date, it has been observed that the general procedure can be used for the determination of only those QAC in which at least one chain is 4 carbons or longer or $C_6H_5CH_2$—or modifications thereof; such compounds will be classified below as Group I. Some examples of Group I compounds that have been determined by the general procedure are: the methobromide of procaine and procaine amide; Banthine; the N-diethylmethyl ethanolamine bromide esters of 1-benzoylcyclopropanecarboxylic acid, of 2,2-diphenylpenten-4-oic, of α-phenoxycinnamic acid, and of 2-(*o*-chlorophenyl)-5,6-dihydro-4H pyran-3-carboxylic acid; the N-(2-hydroxyethyl)-N-methylpiperidinium bromide ester of 1-benzoylcyclopropanecarboxylic acid; the 1-hydroxy-2-(diethylmethylamine) cyclohexane bromide ester of 2,2-diphenylpenten-4-oic; and 4-benzoyl-4-carbethoxy-1,1-dimethylpiperidinium bromide.

Reagents. 1) *Dye solution. 40 mg of bromphenol blue‡ in 50 ml of a 30% K_2HPO_4 solution.* This reagent should be prepared each day. The presence of phosphate-ion was found essential for full color development, the color intensity increasing to a maximum at 0.2 *M* phosphate; excess phosphate-ion had no quenching effects. Therefore, the bromphenol blue reagent was made up in K_2HPO_4 solution in order to keep conditions constant and to avoid unnecessary dilution of the sample. The presence of carbonate, bromide, iodide, chloride, sulfate, acetate, citrate or oxalate ions did not affect the color development in any way. However, the presence of nitrate, even in the presence of phosphate, lessened the intensity of the color development with some compounds but not with others. 2) *Alkaline reagent. 0.8 M borate buffer, pH 9.0 or 20% Na_2CO_3.* The dye complexes of the majority of the compounds tested could be extracted by ethylene dichloride (EDC) from a solution made alkaline with borate buffer, pH 9.0. However, in the case of a few compounds it was necessary to use a stronger alkalinizing agent, 20% Na_2CO_3. 3) *Extracting Solvent. Washed EDC containing 1½% by volume of iso-amyl alcohol.* The EDC used must be washed each week. It was noted that the blue color complex faded very rapidly in EDC not washed this often, but remained stable for at least 1 hour in freshly prepared EDC. The EDC was washed with ⅕ vol. I *N* NaOH, then ⅕ vol. 1 *N* HCl, followed by 3 washes with distilled water. Iso-amyl alcohol, washed

‡ National Aniline Division, Allied Chemical and Dye Corp., Lot No. 14046.

similarly, was added to the washed EDC to minimize any possibility of adsorption of the complex from the solvent onto the glass surface. EDC is the solvent of choice since it does not extract any of the unreacted dye, and reagent blanks read zero against EDC.

Procedure. To a sample, not larger than 2 ml, containing the QAC, 1 ml of appropriate alkali, 1 ml of bromphenol blue reagent and 10 ml of the EDC were added. This was shaken mechanically for 10-15 minutes in a 50 ml glass-stoppered Erlenmeyer flask. The contents of the flask were then centrifuged for 3-5 minutes, at 1500 RPM. The upper layer was aspirated off completely and the lower layer (EDC) was decanted into a dry cuvette and read in a spectrophotometer at 600 mμ against an EDC blank. The samples can be read immediately but the color is stable for at least 1 hour. No blank reading is given by the reagents or normal control media.

Standardization. Standard curves were made for each compound tested. A plot of optical density against the concentration of the QAC yielded straight lines from 2.5 to 50 μg per sample. The lower limit of sensitivity varied but in most cases was around 2-3 μg per sample. With this general procedure it was not necessary to run standards with each determination unless the dye lot was changed. *Recovery.* The recovery of known quantities of a number of compounds from serum or plasma diluted 1-10, buffer solutions, nerve homogenates, and from dilute homogenates of liver, kidney, and heart was 100 ± 2%. When the amount of the QAC in serum or plasma was too small to allow for dilution, it was necessary to make the changes outlined in the next section to obtain complete recovery. The general procedure had to be altered to effect recovery from urine only when the QAC was present in amounts too small to permit adequate dilution of the urine; these changes will be discussed under modified procedures.

Procedure for Group I in undiluted serum (or plasma). With undiluted serum it was necessary to precipitate the proteins before extraction of the QAC. All precipitants tried interfered with the final color development except HPO_3. Therefore the serum was precipitated with an equal volume of 6% HPO_3, allowed to stand for about 10 minutes, and then centrifuged for 15-20 minutes at 2000 RPM. To 3-4 ml of the clear supernatant, 1.5 g Na_2CO_3 and 0.2 g K_2HPO_4 were added, followed by 1 ml of the bromphenol blue reagent and 10 ml of EDC containing, in this case, 3% iso-amyl alcohol. The rest of the procedure was the same as outlined in the general method. The lower limit of sensitivity for the compounds thus determined was about 2 μg per ml of original sample. Standard curves were run for each compound in control serum precipitated with HPO_3. It was found necessary to vary the aliquot of supernatant and/or the amount and kind of alkaline salt used in the determination of small quantities of certain compounds in undiluted serum. For example, $Na_2B_4O_7 \cdot 10\ H_2O$ (1.5 g) was substituted for Na_2CO_3 in the determination of Banthine. Therefore, this procedure is suggested as a guide in the development of a method suitable for the particular compound to be determined. A number of Group I compounds have been recovered from undiluted serum using the above procedure, but difficulties have been encountered in a few instances. Efforts are being continued to develop sensitive procedures for these exceptions.

Modified procedure. The general procedure could not be used for the determination of acetylcholine (ACh) and hexamethonium (C_6), and was found to be 10 times less sensitive for *d*-tubocurarine and neostigmine than for other compounds in Group I. However, for these compounds as well as for tetraethylammonium (TEA), this general procedure has been adapted for use in certain media as shown in Table I. With the exception of the changes indicated in Table I the remainder of the procedure was the same as outlined above for the general method. It is essential to keep the aqueous phase at a minimum for ACh and TEA.

Procedure for undiluted urine. When the amount of QAC in the urine was too small to permit dilution, modifications of the procedure had to be devised. The recovery from undiluted urine varied with specific gravity of the urine tested and with the particular compound determined. All attempts to remove

TABLE I. Examples of Modified Procedures Suitable for Compounds Named in the Media Listed.

Compound	Medium from which extracted	Max aliquot of sample, ml	Alkaline salt used, g	Dye reagent, ml	Total aqueous phase, ml*	% iso-amyl alcohol in EDC	Limit of sensitivity, μg/sample
C_6	Urine (undiluted)	1	1.2 K_2HPO_4 .6 Na_2CO_3	1	5	1.5	2.5
ACh†‡	Ringer's sol. & nerve tiss.	.5	.3 K_2HPO_4 .3 Na_2CO_3	.5	1	4	1
TEA	Ringer's solution	.5–1	.3 K_2HPO_4 .3 Na_2CO_3	.5	1–1.5	4	1.5
	Urine (undiluted)	.5	.4 Na_2CO_3	.5	1	4	2.5
	Serum (diluted 1-10)	1	.8 Na_2CO_3	.5	1.5	4	2.5
Neostigmine	Ringer's solution	1	.4 Na_2CO_3	.5	1.5	4	2.5
	Urine (undiluted)	1	.8 Na_2CO_3	.5	1.5	4	2.5
d-Tubocurarine	Ringer's solution	1	.4 Na_2CO_3	.5	1.5	4	2.5
	Urine (undiluted)	1	.8 Na_2CO_3	.5	1.5	4	2.5

* Where aqueous phase is greater than sum of sample and dye, distilled water was added.
† Period of shaking limited to 10 min. ‡ Lower limit of sensitivity for choline is 15 μg/sample.

the interfering components of urine, either by precipitation, adsorption or extraction, so far, have proved ineffective. These urine components did not interfere by producing a urine blank, but rather prevented the complete recovery of the complex formed between the bromphenol blue and the QAC. For undiluted urine the method was modified in a manner similar to that above, *i.e.*, solid alkaline salts were used. Moreover, in some cases, the addition of 2-3 ml of water as well, improved the degree of recovery. The density of the aqueous phase should be greater than that of the EDC phase. Standards were always prepared in a control urine to insure accuracy. A few typical examples of the modifications used in the procedures for undiluted urine are outlined in Table I, and these may serve as a guide for the development of procedures for other compounds of interest.

Discussion. This method is presented as a general procedure for the estimation of QAC but there is no claim that it is universal. In addition to the compounds already mentioned at least 30 other Group I compounds were determined by this general method. But, as in the case of *d*-tubocurarine, neostigmine, and acetylcholine, there may be other compounds that fall into Group I and yet cannot be satisfactorily determined by the general procedure. The degree of recovery of a compound has also varied from one medium to another; *e.g.*, it is possible to determine small amounts of C_6 in urine with the procedure in Table I, but, as yet, no sensitive procedure has been developed for its recovery from undiluted serum. The reverse is true of Banthine which can be recovered from serum in low amounts but from urine only when present in much larger concentrations. Also, the degree of recovery of some compounds from different samples of urine showed variation. Despite these limitations, the method has been quite useful, and even though, at present, procedures have not been completed for each compound in each type of medium, it is hoped that a report at this time will prove of value.

However, certain generalities seem to be valid, namely that: 1) of the compounds tested so far, only those of the general structure which includes at least one chain of 4 carbons or longer, or $C_6H_5CH_2$—or modifications thereof, can be determined by the general method; 2) under alkaline conditions, no tertiary nitrogen compound has as yet been found which can be determined by the method; 3) there is no blank for the reagents, or normally, for control media.

Moreover, the general method can be used to distinguish Group I compounds from compounds which do not fall into this category. Thus, the general method has proved valuable

in following the metabolism in serum (diluted 1-10) of some of the esters mentioned above (7). The hydrolysis of the ester could be determined without interference from its quaternary metabolite since only those esters were selected for this study which did not form metabolites belonging to Group I.

Summary. A general method is presented for the determination in buffer solutions, nerve homogenates, dilute serum, plasma, and urine of quaternary ammonium compounds in which at least one chain is 4 carbons or longer or $C_6H_5CH_2$—, or modifications thereof. The lower limit of sensitivity, using this procedure, is about 2-3 μg per sample. Adaptations of this method allow for the estimation of these compounds in undiluted serum or plasma. Acetylcholine, hexamethonium, tetraethylammonium, neostigmine and *d*-tubocurarine can be determined in certain media, in concentrations as low as 1-2.5 μg per sample, by means of modifications of the general procedure.

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Anaphylactic Shock in the Pertussis Vaccinated Mouse. II. Diphtheria and Tetanus Toxoids as Antigens.* (19792)

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The observation(1,2) that mice immunized by a mixture of *H. pertussis* organisms and horse serum become markedly anaphylactogenic to a challenging dose of the serum led us to study other antigens. The effect of pertussis vaccination on increasing the sensitizing capacity of diphtheria and tetanus toxoids was particularly interesting because of reported accidents, such as the clinical report(3) of fatal anaphylaxis following reinjection of a pertussis-diphtheria toxoid vaccine.

Methods. The technic utilized was that previously described(1,2). Female, white mice were inoculated intraperitoneally with 0.5 ml of a mixture containing about 8,750 million phase I organisms of *H. pertussis* vaccine, Sharp and Dohme, and 0.1 ml of either diphtheria or tetanus toxoid. The toxoids incorporated in this mixture were as follows: diphtheria toxoid, fluid; diphtheria toxoid, alum precipitated; tetanus toxoid, fluid; tetanus toxoid, alum precipitated.† Control groups of mice receiving only the diphtheria toxoid were injected at the same time as the experimental group. Fifteen days after immunization the mice were challenged by the intravenous injection of 0.1 ml of the respective fluid toxoid. The mortality rate was determined as the number of animals dead after 24 hours/number of animals injected.

Results and discussion. The results are tabulated in Table I. It can be seen that *H. pertussis* vaccine enhances the anaphylactogenicity of fluid diphtheria toxoid but apparently has no effect on tetanus toxoid. It was not deemed necessary to challenge the tetanus toxoid control groups since it had already been observed that the toxoid com-

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† The toxoids used in this study were Diphtheria Toxoid, Illinois Department of Public Health; Diphtheria Toxoid, Alum Precipitated, Lilly; Tetanus Toxoid Fluid, Lilly; and Tetanus Toxoid, Alum Precipitated (Refined), Wyeth.