TABLE I. Acid-soluble Phosphate Compounds in Frozen or Incubated Glands.

Gland	Condition*	P_i	ATP	ADP	AMP
		←——μM/g——→			
Submax	Frozen	2.65	1.24	.74	.58
"	"	3.98	1.46	.92	.42
Parotid	"	2.23	1.09	.46	.40
Lacrimal	"	3.19	1.49	.56	—
Submax	N, 35°C, †	5.81	.50	.30	.26
"	N, 35°C, ‡	3.45	.17	.28	.86
"	N, 1°C, †	3.64	.94	.28	.34
"	N, 35°C, §	5.10	.30	.19	.34
Parotid	N, 35°C	2.87	.30	.30	.18
"	N, 35°C	2.47	.79	.38	.35
"	N, 1°C	2.12	.53	.21	.29
Lacrimal	N, 35°C	6.45	.73	.26	.29
"	N, 1°C	2.45	1.13	.17	.24
Submax	.05 DNP, †	6.32	.45	.47	.56
Parotid	.05 DNP	3.31	.42	.21	.37
Lacrimal	.05 DNP	6.97	.43	.27	.30
Submax	.2 DNP, †	6.84	.09	.12	.33
Parotid	.2 DNP	5.19	.07	.14	.22
Lacrimal	.2 DNP	9.15	.17	.23	.38
Submax	1.0 CN, †	5.81	.02	.09	.18
Parotid	1.0 CN	3.30	.004	.06	.14
Lacrimal	1.0 CN	9.98	.02	.09	.53
Submax	.0 Na, †	4.69	.37	.23	.27
Parotid	.0 Na	2.22	.25	.22	.33
Lacrimal	.0 Na	3.36	.46	.18	.21

* Frozen = immediately frozen; all others incubated 2 hr. N, 35°C = normal medium, 35°C; N, 1°C = normal medium, 1°C; .05 DNP = .05 mM DNP, 35°C; .2 DNP — .2 mM DNP, 35°C; 1.0 CN = 1.0 mM CN^-, 35°C; .0 Na = Na replaced by Tris buffer, 35°C.

† = gland cut perpendicular to shortest dimension.

‡ = gland cut perpendicular to longest dimension.

§ = gland sliced approximately .5 mm thick; lacrimal and parotid glands incubated as removed from the rat.

DNP at 2×10^{-4} M inhibited Ca^{45} transfer activation almost completely whereas 5×10^{-5} M had only slight effect(1). Other procedures which also prevented the increase in Ca^{45} transfer by acetylcholine were tested for their effects on gland adenosine phosphates. Incubation in 10^{-3} M cyanide reduced ATP to 2% of the level after normal incubation whereas incubation in the absence of Na, at least in lacrimal glands, did not reduce the level of ATP below that found in the presence of 5×10^{-5} M DNP. Thus, it appeared that the inhibition of Ca^{45} transport activation produced by Na-lack was brought about by some mechanism other than interference with ATP formation. The effect of incubation at 1°C was also tested and found to have no appreciable effect on the level of ATP as compared to normal incubation at 35°C. Incubation at 1°C inhibits acetylcholine activation of Ca^{45} transfer completely. In this instance, lowered temperature could prevent the utilization of ATP in the transfer process.

Summary. Rat lacrimal and salivary gland pieces up to 2 mm in thickness are able to maintain functional concentrations of ATP *in vitro*. Reduction of ATP levels produced by chemical metabolic inhibitors agrees closely with effects of the same inhibitors on Ca^{45} transfer activation by acetylcholine.

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Iodine-131 Labeling of Purified Microbial Antigens by Microdiffusion. (32425)

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Although the capacity to bind antigen is the fundamental expression of antibody activity, most quantitative serological methods measure only secondary effects of such combination. Accordingly, these methods may provide inaccurate estimates of antigen binding capacity and inadequate tests of the significance of specific antibodies in a

variety of biological phenomena. The ammonium sulfate coprecipitation technique of Farr(1), using antigen trace-labeled with iodine-131, provides a quantitative and sensitive measure of the capacity of antisera to bind protein antigens. Despite the sensitivity and precision of the method(2), it has been virtually neglected by those studying resistance to infection, probably in part because of difficulty in iodinating relatively labile microbial antigens. During a study of the application of coprecipitation serology to certain purified microbial antigens(3), a microdiffusion procedure was employed for trace iodination. This method provided labeled antigens suitable for serological use and should facilitate the application of Farr's method to other antigen-antibody systems.

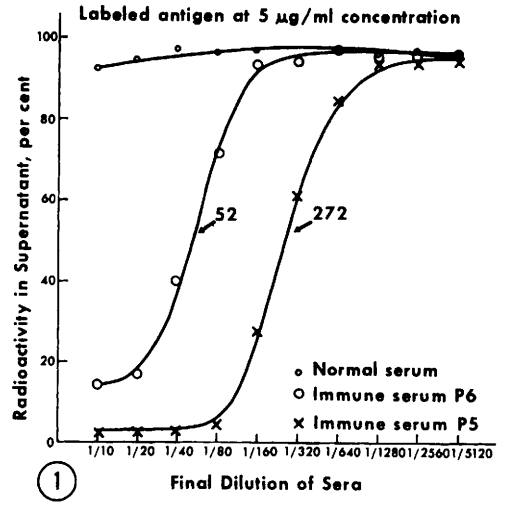
Protective antigen of *Bacillus anthracis*, prepared in our laboratory(4), and enterotoxin B of *Staphylococcus aureus*, kindly supplied by Dr. E. J. Schantz(5), were investigated. Attempts to iodinate the purified anthrax protective antigen by the direct nitrous acid procedure(6) did not yield suitably labeled material that retained serological activity. Use of chloramine T(7) and other procedures also failed to yield suitable material. Because of this difficulty a modification of the microdiffusion procedure of Banerjee and Ekins(8) was investigated. This technique involves transfer of iodine to the protein solution by gaseous diffusion, and eliminates addition of excess oxidizing agents to the protein.

The modification employed was similar to that described by Seth for iodination of serum globulin(9). In place of a Conway microdiffusion cell, a modified 50-ml Erlenmeyer flask was used. A section of glass tubing 2.5 cm high and 1 cm in diameter was sealed to the bottom of the flask, thus forming a separate inner compartment. The protein solution to be iodinated, usually a 1-ml volume, was pipetted into the outer chamber of the vessel. Two-tenths milliliter of 0.002 M KI was pipetted into the central compartment, 50 to 100 μc of I^{131} (as NaI^{131}) was added, and the flask was sealed with a skirt-type vaccine stopper. Stock acid dichromate solution (0.27 M $Na_2Cr_2O_7$ in 36 N H_2SO_4) was diluted 1:20 and 0.2 ml added to the central compartment by means of a syringe with attached 3- or 4-inch needle. A large excess of oxidizing agent must be avoided; otherwise the iodine will be further oxidized to iodate. The flask was held at room temperature (23 to 25°C) and gently rotated occasionally. After one hour the labeled protein solution was withdrawn with another syringe and needle. The free iodide was removed by passage through a Sephadex G-25 column or by dialysis.

In studies with the purified anthrax protective antigen (1 mg per ml concentration) 80% of the radioiodide was converted to gaseous iodine and approximately 20% of the total iodine was transferred to the protein solution. The percentage of the transferred iodine bound to antigen ranged from 10 to 35%. Usually, about 1 atom of iodine was bound per molecule of antigen. Iodination resulting in labeling below the level of 4 atoms of iodine per molecule of antigen did not affect the precipitating activity of the antigen; labeling above the level of 7 atoms per molecule caused appreciable destruction.

In studies with the purified enterotoxin B (2 mg per ml concentration), 70 to 90% of the initial radioiodide was converted to gaseous iodine and approximately 25% was transferred to the toxin. Efficiency of labeling averaged approximately 15% of the transferred iodine. Usually 0.5 to 1.5 atoms of iodine were bound per molecule of toxin. Both an Ouchterlony plate titration and a modified Oudin procedure(10) indicated that essentially all of the precipitating activity was retained after iodination and dialysis.

With both antigens, iodination by microdiffusion resulted in labeled material suitable for use in the ammonium sulfate coprecipitation technique. Tests with the labeled anthrax protective antigen revealed marked differences in the combining capacities of various equine sera (Fig. 1). Hyperimmune pony sera at 1:10 dilution precipitated 86 to 98% of the labeled antigen preparation; only 7% was precipitated by a 1:10 dilution of normal horse serum. Differences in immune sera were evident and were characterized by determining the dilution of serum required to precipitate 50% of the antigen. The reciprocal



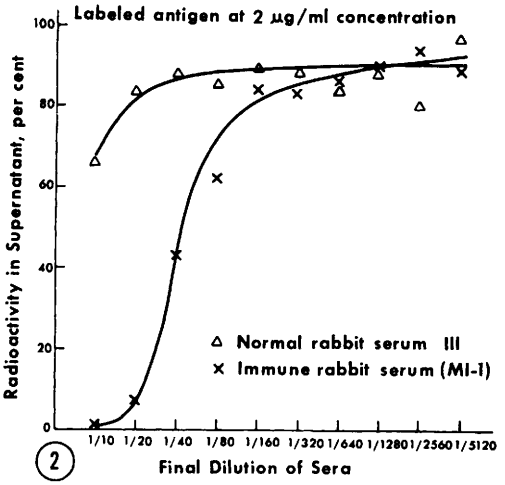


FIG. 1. Coprecipitation of labeled anthrax protective antigen 4048 and equine sera by 1.4 M ammonium sulfate.

FIG. 2. Coprecipitation of labeled enterotoxin B and rabbit sera using 1.6 M ammonium sulfate.

of that serum dilution was taken to represent the titer of the serum. Labeled enterotoxin B also proved suitable for use in coprecipitation serology. Using 1.6 M ammonium sulfate for coprecipitation, differences were found in the combining capacities of normal and immune rabbit sera (Fig. 2).

Summary. Conventional methods for introduction of I^{131} may modify or destroy the serological reactivity of labile microbial antigens. A modified microdiffusion iodination method that eliminates the exposure of antigens to excess oxidizing agent produced satisfactory trace labeling of purified protective antigen of *Bacillus anthracis* and enterotoxin B of *Staphylococcus aureus* without detectable change in precipitating activity. The iodinated antigens proved suitable for use in the ammonium sulfate coprecipitation technique of Farr.

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