All 32 control animals (exposed exactly like the heparinized animals) showed gangrene to the upper border of the exposed area. Nineteen animals were sacrificed between the 4th and 6th day to obtain specimens for histologic studies. They showed, however, clear-cut signs of incipient gangrene and the histologic studies bore out this observation. Of the 51 heparinized animals, 34 had a coagulation time of 30 minutes or more immediately before the next injection was given or, in those on the continuous intravenous drip, twice a day (Table I). Seventeen showed at one or several occasions a coagulation time below 30 minutes or even within the normal range.

Of the 34 animals showing a satisfactory elevation of coagulation time for at least 5 days, 5 developed complete or extensive partial gangrene (15%). Of the 17 animals showing a coagulation time below 30 min. at one or several occasions, 13 developed complete or extensive partial gangrene (76%).

It is thus evident that a continuous satisfactory elevation of the coagulation time of various duration depending on the severity of the lesion is a prerequisite for the successful prevention of gangrene after frostbite. Experiments in which this is not achieved are of no value in judging the merits of the method.

We suggest that the term "heparinization" be reserved only for such applications of heparin therapy where there is proof of a continuously maintained elevation of the coagulation time. The term should not be used for cases in which heparin is injected but proof of a satisfactory result is missing.

*Summary.* 1. Prevention of gangrene after severe frostbite requires a *continuous uninterrupted* prolongation of the coagulation time by heparin injections or infusions for at least 5 days after the exposure.

2. Even brief interruptions of this prolongation lead to a rapid increase in percentage of failure of the treatment.

3. Experiments in which such a prolongation is not achieved continuously are of no value in judging the merits of the therapy.

4. The term "heparinization" should be reserved for such instances in which heparin injections are proved to have produced a continuously maintained elevation of coagulation time to the desired level.

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## Determination of Small Quantities of Nitrogen in Serological Precipitates and Other Biological Materials.* (17791)

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There exists a need for a simple and accurate method for the analysis of nitrogen in microquantities of protein, with particular application to serological precipitates. Pressman(1) and Heidelberger and MacPherson(2) have described quantitative colorimetric methods which employ the Folin-Ciocaltéu phenol reagent(3) and require an empirical calibration curve for converting the phenol color readings to nitrogen or protein. The precision and accuracy of these methods can be readily obtained by the use of Nessler's reagent, with which nitrogen is measured directly as ammonia. The method described below has been employed successfully in this laboratory for the past two years, and certain

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1. Pressman, D., *Ind. Eng. Chem., Anal. Ed.*, 1943, v15, 357.

2. Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, v97, 405; v98, 63.

3. Folin, O., and Ciocaltéu, V., *J. Biol. Chem.*, 1927, v73, 627.

features were previously employed elsewhere (4). In its application to the analysis of serological precipitates, the chief innovation consists in that a given precipitate is prepared, washed, digested, and nesslerized in the same tube; accordingly, the errors involved in transferring precipitates are eliminated, and the time required for the analyses is reduced. At the same time, a gain in sensitivity over the previous method (4) is achieved, without sacrifice of precision, by establishing the final volume of nesslerized material at 7 ml. Since the methods for preparing serological precipitates (5) and the factors affecting the development of color with Nessler's reagent (6) have recently been discussed in detail, these matters will not be considered at length in the present communication.

*Method.* Serological precipitates are allowed to form in Pyrex tubes (100 mm x 13 mm O.D.) and are washed thoroughly by routine methods (5). The tubes are drained over filter paper, and the precipitates, which should contain 5 to 80 γ N, preferably 15 to 60 γ, are treated as follows. A volume of 0.16 ml diluted $H_2SO_4$, prepared by mixing 1 volume concentrated acid (Mallinckrodt Low Nitrogen Analytical Reagent, specific gravity 1.84) and 1.2 volumes distilled water, is added, and the tubes are heated over a hot plate adjusted to allow refluxing of the acid about half to two-thirds of the way up the tubes. Forty-two tubes may be handled as a unit in a special circular brass rack† (Fig. 1) (first introduced by Prof. Dan H. Campbell in collaboration with one of us (4)). The lower plate of the rack was milled out with a ball-shaped mill to increase the area of contact with the tubes. A Meker burner may be substituted for the hot plate as source of heat. In the initial period of heating, water condenses on the cool upper portions of the tubes and may be removed by cautious application of a small flame. After one hour, the rack is allowed to cool for 4 to 5 minutes, and 1 drop 30% $H_2O_2$ (Superoxol, Merck) is added directly to each digest. The tubes are heated again for 5 minutes, cooled and treated with peroxide as before, and heated finally for 10 to 15 minutes. The last traces of water in the upper regions of the tubes may be removed with the use of a small flame. The tubes are then allowed to cool to room temperature.

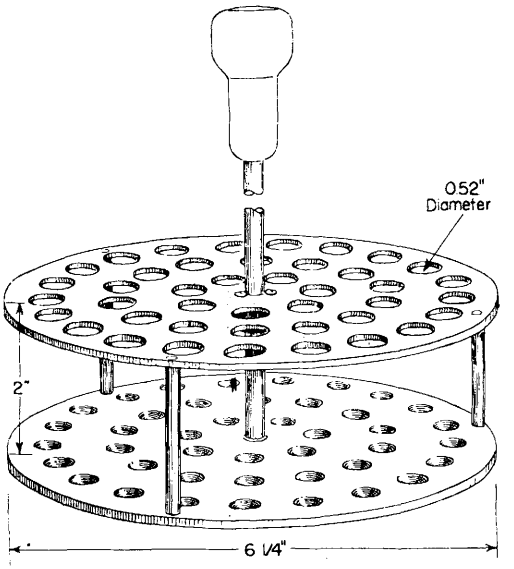


FIG. 1.
Digestion rack. The upper plate is 3/32" thick and the lower plate is 1/8". The handle is a bakelite-capped brass rod 9" long, measured from the bottom plate.

A volume of 5.00 ml distilled water is added to each tube from a buret, and the contents are mixed with a footed glass rod, which is retained. Next, 2.00 ml Nessler's reagent, prepared according to Koch and McMeekin (7)‡, is added rapidly with a pipet, and the contents are mixed immediately with the rod, which is now set aside. A satisfactory method for delivering the Nessler's reagent

4. Lanni, F., and Campbell, D. H., *Stanford Med. Bull.*, 1948, v6, 97.

5. Kabat, E. A., and Mayer, M. M., *Experimental immunochemistry*, Charles C Thomas, Springfield, Ill., 1948.

6. Miller, G. L., and Miller, E. E., *Anal. Chem.*, 1948, v20, 481.

† It has been found desirable to leave some of the places vacant in order to allow sufficient ventilation and thus to avoid excessive heating of the upper regions of centrally located tubes.

7. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, v46, 2066.

‡ The 30 g mercury and 22.5 g iodine recommended by Koch and McMeekin may be satisfactorily replaced by 40.3 g mercuric iodide.

TABLE I.
Accuracy and Precision of Micro-Analytical Colorimetric (Nessler) Method as Tested with Bovine Serum Albumin (BSA) and Egg-White (EW).

| Protein solution* | Date | Replicates by Nessler method, γ N | | | Avg, γ N | Expected,† γ N | % accounted for | Avg dev. of replicates, % |
|---|---|---|---|---|---|---|---|---|
| BSA-1 | 10/15/47 | 28.0 | 28.1 | 29.2 | 28.4 | 28.8 | 98.6 | ±1.8 |
| " | " | 55.8 | 58.4 | | 57.1 | 57.6 | 99.1 | 2.3 |
| " | 11/ 1 | 27.1 | 27.5 | 27.6 | 27.4 | 28.8 | 95.1 | 0.7 |
| " | " | 53.9 | 55.6 | 56.7 | 55.4 | 57.6 | 96.2 | 1.8 |
| "-2 | 5/ 8/48 | 30.1 | 30.9 | 31.1 | 30.7 | 30.9‡ | 99.4 | 1.3 |
| " | " | 61.2 | 62.3 | | 61.8 | 61.8‡ | 100.0 | 0.9 |
| " | 10 | 30.2 | 31.1 | | 30.6 | 30.9‡ | 99.0 | 1.5 |
| " | " | 60.8 | 62.8 | | 61.8 | 61.8‡ | 100.0 | 1.6 |
| "-3 | 5/12/49 | 32.4 | 32.6 | 33.0 | 32.7 | 33.7 | 97.0 | 0.7 |
| " | 20 | 52.0 | 52.4 | 53.6 | 52.7 | 56.2 | 93.7 | 1.0 |
| "-4 | 20 | 52.9 | 53.8 | 54.2 | 53.6 | 53.4 | 100.4 | 0.9 |
| " | 25 | 52.8 | 53.4 | 54.0 | 53.4 | 53.4 | 100.0 | 0.7 |
| " | 6/ 2 | 51.2 | 51.6 | 51.6 | 51.5 | 53.4 | 96.4 | 0.3 |
| " | 10 | 51.2 | 51.6 | 53.2 | 52.0 | 53.4 | 97.4 | 1.5 |
| "-5 | 12/ 9 | 18.7 | 19.7 | 20.1 | 19.5 | 20.2 | 96.5 | 2.7 |
| " | 2/ 2/50 | 37.2 | 38.2 | 38.7 | 38.0 | 40.5 | 93.8 | 1.5 |
| EW-1 | 11/17/48 | 30.8 | 32.0 | | 31.4 | 31.4 | 100.0 | 1.9 |
| "-2 | 11/17/48 | 27.4 | 27.4 | | 27.4 | 28.7 | 95.5 | 0.0 |
| "-3 | 2/24/50 | 20.3 | 20.4 | 21.6 | 20.7 | 21.0 | 98.6 | 2.6 |
| Avg | | | | | | | 97.7 | ±1.4 |

* Aliquots of 0.50 to 2.00 ml of suitable dilutions were dried in Pyrex tubes as described in the text.
† According to determinations by semi-micro titrimetric Kjeldahl method carried out at about 1.0 mg N.
‡ 31.2 and 62.4 γ N expected according to Van Slyke gasometric determination.

consists in using a 2-ml serological pipet firmly clamped in vertical orientation to a stand. The upper end of the pipet is connected by rubber tubing to a 5-ml syringe, which is mounted at table level. The reagent is alternately drawn up into the pipet from a reservoir and emptied with moderate force directly into a diluted digest. The volume thus delivered, while not exactly 2 ml, is sufficiently constant for accurate quantitative work. The nesslerized sample is transferred to a colorimeter tube, and the optical density is determined after a timed interval, usually 60 seconds, with a suitable photoelectric colorimeter, *e.g.*, the Evelyn colorimeter, used in our work in conjunction with the No. 515 filter. Longer periods of time, at least up to one hour, may be allowed to elapse before the readings, provided that blank and standard readings are made after the same interval.

Blanks, devoid of protein, are carried along with the unknown samples through the entire process of digestion and treatment with peroxide. Some of the blanks are later converted to standards by the addition of 4.00 ml, instead of 5.00 ml, $H_2O$ and 1.00 ml standard $(NH_4)_2SO_4$ solution (preserved with 1.4 ml concentrated $H_2SO_4$ per liter) containing 5 to 80 γ N per ml. A standard curve, covering the expected range of nitrogen values, is prepared with each run. Such standard curves, in which optical density is plotted against quantity of nitrogen, are sufficiently linear that they may be regarded as such in the evaluation of the results(6). The above method is readily adapted to the analysis of protein solutions by the simple expedient of drying down a suitable volume of solution at 90 to 95°C in a Pyrex tube of the size indicated above.

*Results.* Table I illustrates the accuracy and precision of the method. Stock solutions of bovine serum albumin and egg-white, containing about 1.0 mg N per ml, were analyzed by a semi-micro titrimetric Kjeldahl method to provide reference values. Aliquots of suitable dilutions were analyzed by the colorimetric method. On one occasion a stock solution of bovine serum albumin was analyzed also by a Van Slyke gasometric procedure. The colorimetric method yielded, on the average, 97.7% of the nitrogen expected from

TABLE II.
Precision of Determination of Amounts of Nitrogen Precipitated from Duplicate Mixtures of Purified Swine Influenza Virus and Convalescent Swine Influenza Antiserum.

| Duplicates γ N | | Avg dev., % | Duplicates, γ N | | Avg dev., % |
|---|---|---|---|---|---|
| 4.0 | 4.5 | ±5.9 | 21.0 | 21.8 | ±1.9 |
| 6.0 | 6.4 | 3.2 | 22.1 | 23.4 | 2.9 |
| 7.0 | 7.5 | 3.4 | 23.0 | 23.9 | 1.9 |
| 7.4 | 8.7 | 8.1 | 24.0 | 24.6 | 1.2 |
| 8.6 | 8.8 | 1.1 | 24.0 | 25.3 | 2.6 |
| 10.2 | 10.6 | 1.9 | 25.0 | 25.4 | 0.8 |
| 9.8 | 11.5 | 8.0 | 25.1 | 25.3 | 0.4 |
| 12.8 | 13.1 | 1.2 | 26.6 | 27.0 | 0.7 |
| 13.9 | 14.1 | 0.7 | 27.4 | 27.4 | 0.0 |
| 12.8 | 15.3 | 8.9 | 27.3 | 27.9 | 1.1 |
| 14.6 | 14.9 | 1.0 | 27.3 | 28.0 | 1.3 |
| 15.2 | 16.2 | 3.2 | 26.8 | 28.9 | 3.8 |
| 15.4 | 16.2 | 2.5 | 27.3 | 28.4 | 2.0 |
| 15.6 | 16.1 | 1.6 | 30.7 | 31.4 | 1.1 |
| 15.8 | 17.2 | 4.2 | 34.6 | 35.0 | 0.6 |
| 17.1 | 17.4 | 0.9 | 35.8 | 37.5 | 2.3 |
| 17.1 | 19.5 | 6.6 | 39.3 | 40.2 | 1.1 |
| 18.4 | 20.0 | 3.2 | 45.8 | 46.2 | 0.4 |
| 20.4 | 20.4 | 0.0 | 45.0 | 49.3 | 4.6 |
| 20.0 | 21.1 | 2.9 | 50.0 | 50.8 | 0.8 |

the titrimetric analysis; the average deviation of replicate determinations was about ± 1.4%. The yield with respect to the Van Slyke procedure was 98.6%. Investigation showed that approximately 1% of the digest was lost by spattering during each of the 2 treatments with peroxide. With a correction for these losses, the accuracy of the microanalysis closely approaches 100%.

The precision of the method for the determination of nitrogen in serological precipitates is illustrated in Table II, which shows results obtained with washed precipitates from mixtures of purified swine influenza virus and convalescent swine influenza antiserum. The errors in these analyses arise from the formation and handling of the precipitates, as well as from the nitrogen determination. It is evident that the overall precision is compatible with the demands of quantitative serological investigations. Results similar to those of Table II have been obtained with mixtures of SII, the type-specific capsular polysaccharide of Type II pneumococcus, and homologous horse antiserum.

The method is not intended to replace the more precise and accurate titrimetric methods, but is regarded as useful for the analysis of small samples, costly samples, or large numbers of routine samples(8-11) whenever the indicated errors can be tolerated. Experience has shown that its useful range extends from about 5 to 80 γ N.§

*Summary.* A simple and accurate colorimetric method is described for the routine determination of quantities of nitrogen in the range from 5 to 80 γ. The method, which is a modification of the direct-nesslerization procedure of Koch and McMeekin, has been developed especially for the analysis of serological precipitates, but is adapted readily to the analysis of protein solutions.

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