A sensitive protein assay method using micro-titer plates

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Summary. A commercially available protein-assay, based on the reaction between bicinchoninic acid (4,4'-dicarboxy-2,2'-biquinoline) and copper was adapted for use in microtiter plates. The assay is sensitive between 10 µg/ml and several mg/ml, and it uses only 10 µl of sample material. The results obtained with the method were in accordance with other protein determination methods. The assay was shown to be reproducible, reliable and insensitive to non-ionic detergents.
Key words. Bicinchoninic acid; protein-determination; non-ionic detergents; micro-titer plate.

A number of methods are currently available for determining the concentration of proteins in biological and biochemical samples. A new method has recently been made commercially available by Pierce Chemicals[2]. The method relies on the fact that proteins are able to catalyze the reduction of Cu^{2+} to Cu^{+} which then forms a colored complex with bicinchoninic acid. This method is highly sensitive and easy to perform but requires exact timing. We have adapted this assay for use in micro-titer plates; the method allows the determination of hundreds of samples per day.

Materials and methods. The 'BCA' reagents were from Pierce; the protein used for determination of the standard curve was bovine serum albumin from Sigma, MO, USA; Triton X-100 and Tween 20 were from Fluka, Buchs, Switzerland.

The micro-titer plates were the polystyrene ones from NUNC Denmark, product code 2-69620. These plates are designed for spectrophotometric determinations of color-reactions (i.e. non-immune applications).

Samples, 10 µl each, were pipetted into individual wells to which 200-µl aliquots of 'BCA'-reagent were added using an 8-channel pipette. A separate mixing step was not necessary since this was achieved by the addition of the reagent itself. After incubation for various times at different temperatures the plates were read in a Flow MC Elisa-reader at 540 nm (standard filter No. 6). Wavelength-scans were performed on an LKB Ultrospec 4050 photometer. All instruments were interfaced to a Commodore C64 microcomputer, which handled all data and also performed plotting with the Watanabe Graphtec MP1000 plotter.

Results and discussion. According to the manufacturers' specifications, measurements should be performed at 562 nm. This wavelength is not normally available on photometers for microtiter plates. However, as shown in figure 1, the use of the common 540 nm filter is permissible and yields an extinction coefficient which is 0.89 times that obtained at 562 nm.

Figure 2 shows the relation between the protein concentration and color yield at a) three different temperatures and b) after

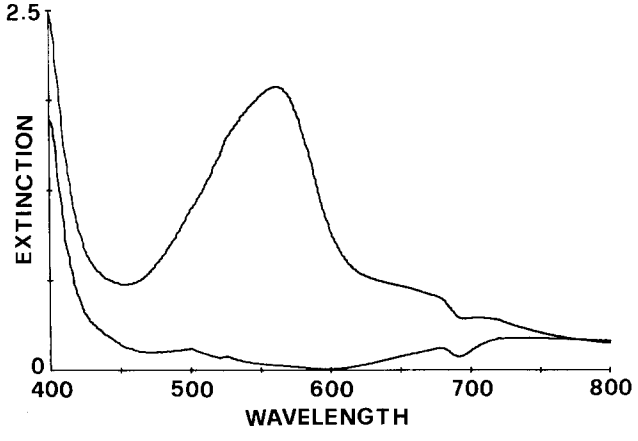

Figure 1. Wavelength scans of the reagent blank (bottom curve), and the formed Cu^{+}-bicinchoninic acid-complex (top curve).

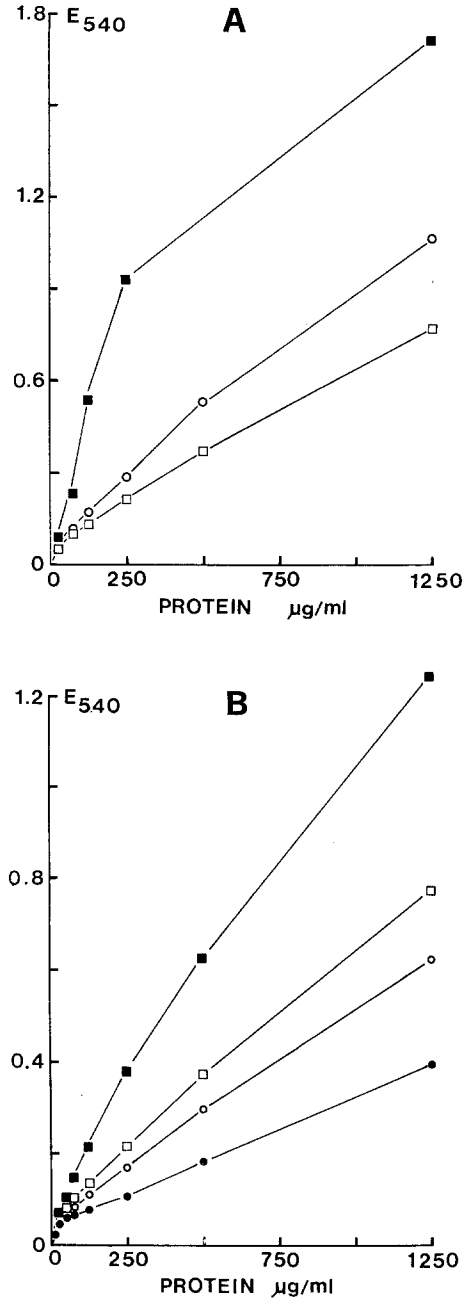

Figure 2. Dependence of color yield on protein concentration with A) different temperatures and B) different reaction times. *A* Determinations were performed at 20 °C (□), 37 °C(○), and 60 °C. *B* At 20 °C the reaction was allowed to proceed for 20 min (●), 1 h (○), 2 h (□) and 20 h (■).

different reaction times. Since the color yield depends on a catalyzed reaction between protein and the assay reagents, an increase in temperature will increase the color yield at a given incubation time. We have tested the color yield at room temperature, 37 °C and 60 °C, and found that temperatures above 37 °C gave unsatisfactory results for the following two reasons; firstly, heating of the micro-titer plate caused uneven evaporation leading to erroneous results, and secondly, Triton X-100 clouded which resulted in a two-phase system.

Prolongation of the reaction time will also give an increase in color yield. In our hands optimal results were obtained when the plates were first incubated at room temperature for 20 min. If the unknown samples had reacted, i.e. if a color formation could easily be seen, the plate was read in the photometer. This was typically the case for samples with protein concentrations above 200 µg/ml. If the wells contained less protein (down to 10 µg/ml) the plate was either kept at room temperature for 20 h or at 37 °C for 2 h, and finally measured spectrophotometrically again. This two-step reading allows to the very rapid determination of protein concentrations in the range of 10 µg/ml to 2 mg/ml.

The influence of the non-ionic detergents Triton X-100 and Tween 20 was investigated since these additives are common in studies involving membrane proteins. The standard curves for protein in the concentration range of 10–1500 µg/ml was not influenced by either detergent in concentrations up to 5% (the highest measured). In all cases the standard curves could be superimposed on those obtained without detergents, i.e. within the standard error of the assay (± 5%). Nonetheless, we recommend that the standard curve be recorded in the presence of the same detergent at the same concentration as in the unknown sample, since some detergents may have (oxidative) impurities which could affect the determination. Since there was no indication of a concentration dependency it is likely that even higher detergent concentrations will not interfere. We have further compared the values obtained with our modified BCA assay to those obtained by the method of Lowry, as modified by Wang and Smith[3], a method which also excludes interference from non-ionic detergents. Detergent extracts from animal brains were analyzed for protein using both methods, and in all cases the results differed by less than ±10%.

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2 Pierce Product Information, No 23225.

3 Wang, C.-S., and Smith, R.L., Analyt. Biochem. *63* (1975) 414.

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Acute cold exposure increases the glucagon sensitivity of thermogenic metabolism in the rat

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Summary. Administration of glucagon to rats at 25 °C had no effect upon their $\dot{V}O_2$, while administration of noradrenaline or noradrenaline plus glucagon raised the $\dot{V}O_2$. At 5 °C, noradrenaline had no effect upon the cold-enhanced $\dot{V}O_2$, while glucagon caused a rise of 13.7%, implying increased glucagon sensitivity at 5 °C. The glucagon-induced enhancement of $\dot{V}O_2$ was abolished by concurrent administration of noradrenaline.
Key words. Glucagon; thermogenesis; cold-exposure; $\dot{V}O_2$.

On exposure of animals to low environmental temperatures, thermogenic metabolism is increased in order to maintain body temperature[1]. Upon initial exposure, the primary method of additional heat production is that of shivering; with increased duration of cold exposure, non-shivering mechanisms, primarily attributable to brown adipose tissue (BAT) become predominant[2] and eventually replace shivering as the source of enhanced thermogenesis[3]. Activation of BAT is widely considered to result from increased adrenergic activity during cold exposure[4]. There is substantial evidence, however, that non-adrenergic factors may also be implicated in the activation of BAT[5]. Such factors may include the corticosteroids[6] and glucagon, the latter of which has been suggested to be an important component of a multifactorial activation of BAT in cold environments[7]. BAT appears to be active within 1 h of cold exposure[8], as evidenced by increased mitochondrial GDP-binding, indicating raised levels of 32 kdalton uncoupling protein[9]. Therefore, as corticosteroids are generally regarded as relatively slow-acting agonists, while plasma glucagon has been shown to be significantly elevated after cold exposure for 1 h[10], the experiments described below were undertaken to obtain an indication of the relative potencies of noradrenaline and glucagon as promoters of thermogenic metabolism. The potencies were assessed by measurement of oxygen consumption ($\dot{V}O_2$) of intact animals at thermoneutrality and in acute cold exposure.

Materials and methods. Male Wistar albino rats of body weight 210 ± 6.8 (SEM) g were used. They were held at an ambient temperature of 22.5 ± 1.0 °C, with a 12 h photoperiod (08.00–20.00 h), and received food and water ad libitum. The animals were divided into 2 groups: one for experiments at thermoneutrality (25 °C) and one for experiments in acute cold exposure (5 °C). Neither group had previous experience of cold exposure. All experiments were performed between 10.00 h and 16.00 h, at the nadir of their circadian cycle as indicated by plasma corticosterone concentration[11]. Four treatment regimes were employed at each experimental temperature: 1) Control, consisting of the vehicle used in drug administrations (NaCl 250 µg/ml and lactose 1.07 mg/ml in sterile distilled water, pH adjusted to 3.5 with 0.1 M HCl) administered at a dose of 0.1 ml/100 g b.wt, i.p.; 2) Noradrenaline as the acid tartrate (Levophed: Winthrop Laboratories, U.K.), was diluted with the vehicle to 250 µg/ml and given at a dose of 25 µg/100 g b.wt, i.p.; 3) Glucagon (Novo Industri A/S, Denmark) as the hydrochloride at 1 mg/ml in the vehicle, given at 100 µg/100 g b.wt, i.p. (12); and 4) Noradrenaline plus glucagon at the doses indicated above, given in a total volume of 0.1 ml/100 g b.wt, i.p. Immediately following administration of the drug or vehicle, the animals were placed individually into the temperature-controlled chambers of a high-precision respirometric system as previously described[13]. Measurements of $\dot{V}O_2$ were noted at 10-min intervals during the first hour after the administration of vehicle or drugs. Data from the second hour were expressed as ml O_2/min/kg$^{0.75}$ at standard temperature and pressure of dry gas. Data from the second hour were used because elevations of $\dot{V}O_2$ following handling and