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ZONAL IMMOBILIZATION OF PROTEINS

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SUMMARY A gel matrix that can be used in sequence to separate proteins and then immobilize them was obtained by incorporating into agarose an aldehydic ligand with readily controllable reactivity. The gel was prepared by etherifying agarose with glycidol and subsequently oxidizing with periodate. It provided an inert matrix equivalent to ordinary agarose for separating proteins at neutral or acidic pH, but rapidly absorbed them through formation of stable alkyl amine linkages on exposure to either alkaline or concentrated $NaCNBH_3$. Thus, the protein could be fixed without use of denaturants. The ability to array proteins electrophoretically on an immobilizing substrate opens new possibilities for analysis of complex mixtures by providing means for carrying out affinity binding assays in relation to physical properties of the protein, and for performing multiple tests of composition without loss or spread.

To improvise a system for analyzing electrophoretic separations that would incorporate advantages of processing the separated material in covalently immobilized form, we sought to devise a gel support that could be used in sequence as both a separation and an immobilizing medium. This communication describes an aldehydic derivative of agarose which we have found to be well suited for such use (1, 2).

Studies by Borch et al. (3) indicated that many alkanals did not interact appreciably with amines at neurtal pH, but could be rapidly driven to form stable alkyl amine linkages by admixing sodium cyanoborohydride ($NaCNBH_3$) as a highly specific and efficient Schiff-base reducing agent. We inferred that an appropriate aldehydic substituent could be incorporated into agarose without altering utility of the agarose as an electrophoretic meduim, while adding facility to fix the protein under nondenaturative conditions upon exposure to $NaCNBH_3$. A derivative that we felt might be applicable had been prepared by Porath and Axén (4) by treating agarose with epichlorohydrin and then oxidizing with periodate; however, crosslinking of the gel by the epichlorohydrin imparted undesirable rigidity. On substituting glycidol for epichlorohydrin, we obtained an aldehyde-rich gel that

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could be melted for casting films as usual for electrophoresis. We call the gel "glyoxyl agarose" to specify its derivation (eqn. 1) as oxidized glyceryl agarose.

$$\text{Agarose} + \underset{\text{O}}{\text{CH}_2\text{CHCH}_2\text{OH}} \xrightarrow{\text{OH}^-} \text{Agarose-O-CH}_2\text{CHOHCH}_2\text{OH} \xrightarrow{\text{IO}_4} \text{Agarose-O-CH}_2\text{CHO} + \text{HCHO} \quad (1)$$

MATERIALS AND METHODS

Glyoxyl Agarose. A 200 ml suspension (170 ml bed) of 50-100 mesh 4% agarose gel (Bio Gel 15 m, Bio Rad Laboratories) was washed with water, and admixed with experimental quantities (12 ml recommended) of glycidol (Eastman Chemical Co.), and 100 ml of 1M NaOH containing $NaBH_4$ (2 mg/ml) as antioxidant (4). The mixture was stirred by rocking for 18 h at ambient 25°C, and then washed with water through a Büchner funnel with only slight suction until pH fell to 7 ± 0.5. The resultant glyceryl agarose was suspended in water to 250 ml, then admixed with 70 ml of 0.16M $NaIO_4$, and stirred by rocking for an hour. The aldehyde content was determined as equivalent to the periodate consumed. The gel was then washed free of formaldehyde by-product and periodate, and stored in aqueous suspension at 4°C with volume adjusted to equal four-thirds of the bed volume, so that unit volume of suspension would by calculation contain 2 percent agarose.

Electrophoretic Gel. The glyoxyl agarose was usually diluted with regular agarose by melting together 1 g of agarose powder, plus 37 ml of 0.03M sodium phosphate at pH 6.85, plus 12.5 ml of the 2% glyoxyl agarose suspension, plus 0.5 ml of either 10% SDS or buffer. This formulation yielded gel with 2.5% agarose content overall (glyoxyl/ ordinary = 1:4), a composition found suitable for separation of proteins with molecular weights ranging from 10^4 to 10^6 Daltons. For electrophoresis at pH 10, the gel was prepared at neutral pH to avoid heating in alkali, and then washed with 0.03M sodium carbonate. The gels were cast at 1.5 mm thickness.

RESULTS

Langmuir plots of reaction isotherms over a range of ambient temperatures enabled us to derive an equation (Fig. 1) for predicting amounts of glycidol required for specified aldehyde content. Linearity of the isotherms indicated that the derivatization involved uniform sites which from maximal yield (1.5 ± 0.1 meq/g) corresponded to one site per two biose (4) units. Except where indicated, gel containing 1 to 1.2 meq aldehyde per g was employed for electrophoresis because of ease of melting and high protein binding efficiency.

Protein binding by the gel proceeded as anticipated by being negligible at pH <8.4. Rapid uptake of protein occurred at pH 10, as indicated by experiments in which test proteins were electrophoretically transferred through ordinary agarose into an abutting film of glyoxyl agarose where the protein would accumulate in a concentrated band. Such experiments showed (Fig. 2) that a high content of aldehyde

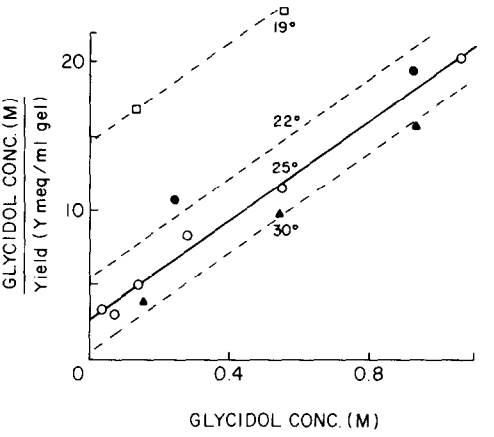

Figure 1. Reaction isotherms. Periodate consumed in oxidation of the gel (Y meq/ml) appeared to vary as a reciprocal function of the molarity (M) of glycidol used for etherification, as in hyperbolic relationship. From the slope (16.9 ml/meq = 1/maximal yield) and intercept (2.55) at 25°C, Y=M/(2.55 + 16.9M). Isotherms for reactions with glycidol at 19°C and 30°C appeared to have similar slopes, and their intercepts $(M/Y)_o$ varied in approximate logarithmic proportion to $1/K^o$ as though thermodynamically related to an exothermic equilibrium in the reaction. Thus, intercepts for prediction of yields at ambient temperatures other than those studied may be approximated from the equation: $\text{Log } (M/Y)_o = \text{Log } 2.55 - 1.12 \times 10^4 (1/298 - 1/K^o)$. No volume change was incurred in modifying the agarose. Since spherulated 4% agarose gel was used, the aldehyde content per ml bed was calculated as two thirds that of the gel itself, and content per g (dry) of the original gel matrix equalled 25Y. Measurements of dry weights per unit volume of the original agarose and of the product agreed with expectation that modification to a level of 1 meq/g added only a small (6 ± 2%, N = 4) increase in solid content of the gel matrix.

≤1 mmole/g (dry) was essential for rapid immobilization. Further, only moderate quantities of protein on the order of 1 mg per ml of gel could be absorbed with high efficiency, regardless of the concentration of glyoxyl agarose in the gel. This level of uptake of numerous test proteins was achieved with a gel mixture contianing 0.5% glyoxyl and 2% regular agarose, and did not increase measurably on enriching the glyoxyl agarose to 1.5%, the total agarose (glyoxyl + regular) being held constant at 2.5%.

Much of the binding achieved by the alkaline gel could be reversed by either neutralizing or adding amine-rich buffer, but binding became irreversible when 0.02M $NaCNBH_3$ was added after which neither urea, amines, SDS, or monochloroacetic acid could displace the protein. Full immobilization required an hour when the alkaline gel was simply immersed in the $NaCNBH_3$, but required only 15 mins when the $NaCNBH_3$ was added electrophoretically.

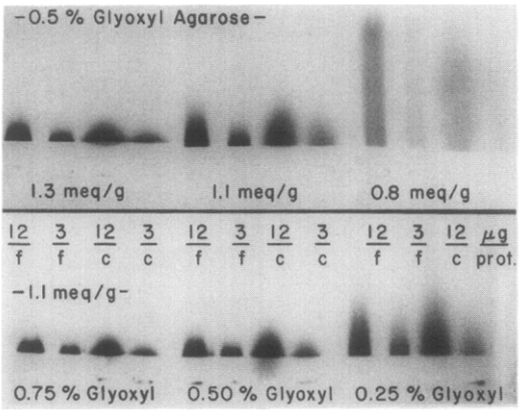

Figure 2. Protein binding characteristics of glyoxyl agarose at pH 10. Fibrinogen (f) and cytochrome C (c) were applied at 12 µg and 3 µg levels in 1 x 5 mm slots in regular agarose, and were transported (upwards) at 8 volts/cm into adjoining gel containing glyoxyl agarose which retained the protein. The efficiency of protein absorption was assessed from the volume of gel traversed by the protein up to the point of complete uptake. The lower panel shows effect of varying the concentration of glyoxyl agarose by diluting with regular agarose, the overall gel concentration (glyoxyl + regular) being held constant at 2.5%. The glyoxyl agarose preparation contained 1.1 meq aldehyde /g. With the diluted gel, the binding achieved per unit weight of glyoxyl agarose approached that observed in batch-uptake experiments with undiluted gel (0.12-0.15 mg/mg glyoxyl agarose). The absorptive capacity per unit volume of gel increased only by a factor of two on increasing glyoxyl agarose concentrations above 0.5% (to 2.5% not shown), but decreased in a proportionate way below 0.5%. Despite higher capacity per unit volume of concentrated gel, uptake of protein at levels exceeding 1 mg/ml proceeded slowly. Rapid uptake to a level of 1 mg/ml by 0.5% glyoxyl agarose was evident from the uniformity of staining of areas containing absorbed protein, the volume occupied per µg of protein, and the sharpness of the boundaries demarcating depletion of the protein. The upper panel shows effect of varying the aldehyde content of the glyoxyl agarose, the concentration being held constant at 0.5%. The absorptive efficiency decreased substantially on reducing the aldehyde content of the glyoxyl agarose matrix to 0.8 meq/g, and improved somewhat on raising the aldehyde content to 1.3 meq/g. The buffer used in this experiment contained admixed Lubrol (1%) and SDS (0.1%). The admixed detergents had noticeable inhibitory effect in that uptake of the protein by glyoxyl agarose containing 1.1 meq aldehyde/g was only half that observed in the lower panel where the detergent was omitted.

At pH <8.4, plasma proteins traversed the gel without trailing. Further, no uptake of the protein was discernible after 2 days exposure to the gel. However, a full uptake (>98%) of protein at subsaturating concentrations (<1 mg/ml) commenced over a 1 h period on adding 40 mM $NaCNBH_3$ to the medium at pH 6 or 2 h at pH 7.4. The protein uptake occurred only while $NaCNBH_3$ was present, and ceased when $NaCNBH_3$ was removed. The effect of $NaCNBH_3$ appeared as anticipated (3) to be limited to linkage of the protein to the gel; it was found to have no effect on either solubility or thrombin-coagulability of fibrinogen over a 24 h period in absence of the gel, nor did it alter the gel in absence of the protein.

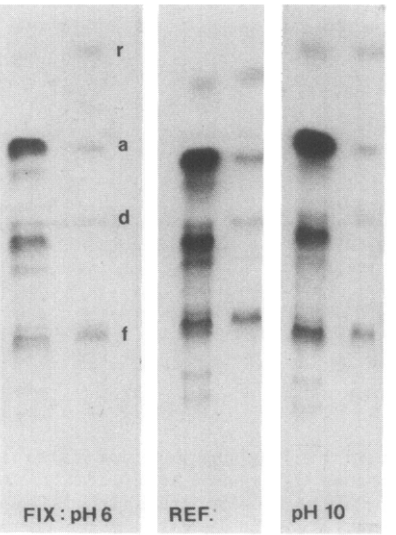

Figure 3. Band patterns obtained after electrophoretically separating test proteins and plasma on glyoxyl agarose in neutral phosphate with 0.1% SDS, and fixing them either by covalent immobilization with 0.02M $NaCNBH_3$ at pH 10 or 0.1M $NaCNBH_3$ at pH 6 (1% Lubrol also added), or by precipitation with 20% sulfosalicylic acid for reference. Test proteins: 4 µg human fibrinogen (f) 4 µg bovine serum albumin monomer (a) and dimer (d), and 2.5 µg bovine pancreatic ribonuclease-A (r). Plasma: 2 µL at 1:5 dilution.

Addition of SDS (0.1%) seriously impaired both the rate and extent of absorption of protein by the gel whether at pH 10 or with $NaCNBH_3$ added, but the inhibitory effect could be abolished by adding Lubrol$^{(R)}$ or Triton$^{(R)}$ (5, 6). Thus as illustrated (Fig. 3), plasma proteins once separated by electrophoresis in SDS became fixed in bands as sharp as with conventional fixation by simply immersing the gel for 2 h in either alkaline 0.02M or neutral 0.1M $NaCNBH_3$ containing 1% Lubrol. With the protein covalently attached to the gel, it could be washed and stored in either neutral buffer or SDS solution for a month without loss of the protein bands. When exposed to labelled anti-fibrinogen antibody, uptake of antibody was seen only in the appropriate position for fibrinogen. Further, the absorbed antibody could be desorbed with either buffer at pH 2.4 or with 0.1% SDS. As will be detailed (1), quantitative measurement of the desorbed antibody indicated that two-thirds of the anigenic determinants in the immobilized fibrinogen remained accessible to the antibody.

DISCUSSION

The development of an immobilizing gel that can be used to separate proteins before immobilizing them presents a new research tool. Glyoxyl agarose may be used either alone or diluted with ordinary agarose within the recommended limits, or as a composite (7) with polyacrylamide gel. We find it advantageous to prepare composites with degradable polyacrylamide (8). When proteins are separated and immobilized on the composite gel, they become attached only to the glyoxyl agarose matrix, a condition allowing removal of the polyacrylamide without effect on the protein distribution.

Glyoxyl agarose offers advantage as an electrophoresis medium because of the ease of fixing protein on it and because the fixation by immobilization does not depend on denaturation. However, we view ability to subject the immobilized protein (9) to multiple analyses without risk of dissolution or spreading as the unique advantage. A subsequent communication (1) will detail a new immunoelectrophoresis procedure which utilizes multiple processing in a cascaded fashion.

Conceptually, the ability to array proteins on an immobilizing substrate in positional relation to the physical properties of the protein may be viewed as adding a new dimension to immobilization technology.

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