## Separation of specific fractions of synaptosomes by affinity chromatography

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*Summary.* The authors describe a new technique for isolation of specific fractions of synaptosomes, on the basis of their surface glycoproteins, by affinity chromatography using lectin-Sepharose columns.

The use of affinity chromatography has made possible the separation of diverse types of cells, including neurons from sympathetic ganglia[1]. In this note, we describe the possibility of separating different types of brain synaptosomes by affinity chromatography using lectins.

Concanavalin A (Con A) and other lectins are capable of binding to the synaptosome surface[2-6]. By means of affinity chromatography using lectins, diverse glycoproteins fractions can be separated from whole brain or synaptic plasma membranes[6-10]. The existence of these synaptic plasma membrane glycoproteins capable of interacting with lectins[6,8,10] may serve to separate different types of synaptosomes. Histochemical studies have demonstrated that Con A does not bind to all synaptosomes nor to all synapses in sections from nervous system[6]. Therefore, it should be possible in theory to separate the synaptosomes interacting with Con A from these not interacting. This is also possible in practice. The use of a column of Con A coupled to Sepharose-4B is one way to achieve it. The technique used by us is as follows:

A) Isolation of a synaptosome fraction from brain of rat or mouse, as the technique of Morgan et al.[11]. B) The fraction obtained in A is filtered through a column of Sepharose-4B-200. C) The filtrate resulting in B is filtered through a column of Sepharose 4B coupled to Con A (purchased from Sigma Chemical Co). The sample is eluted with PBS (phosphate buffer saline: 0.14 M NaCl, 2.7 mM KCl, 16.2 mM $Na_2H\ PO_4$, 1.5 mM $KH_2PO_4$) pH 7.4. In this way, the fraction I of synaptosomes not-interacting with Con A is obtained. The fraction retained by the column is eluted with 0.25 M alpha-methyl-glucoside. This fraction is collected and constitues the fraction II of synaptosomes interacting with Con A. The fraction is centrifugated (11.500 × g, 25 min) and the pellet resuspended in PBS. This is repeated twice or thrice more. After washed in this way with PBS, the synaptosomes are able of interacting again with a column of Con A coupled to Sepharose, which shows that the alpha-methyl-glucoside has been removed. The washed synaptosomes can be liophilized and kept for further studies. The purity of the fractions obtained depends on the purity of the fraction obtained in A. It is also possible to start from whole brain homogenates and so the step A is eliminated. In this case we obtained highly contaminated fractions but useful for morphological studies. Purity of the fractions was controlled by electron microscopy.

The technique described here can be carried out with other lectins, and so it is possible to obtain diverse fractions of synaptosomes on the basis of their predominant membrane glycoproteins. This technique can be used for synaptosomes as well as for other subcellular fractions, such as nuclei which have Con A receptors at membrane level[6,12].

Affinity chromatography may be a useful technique to separate specific synaptosomes, not only because of their capacity of interacting with lectins, but also due to the presence of another type of surface antigens. Perhaps in this way, the synaptosomes might be separated by the function of their different synaptic receptors.

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## Factors influencing the distribution of 99m-technetium methylene diphosphonate in bone and soft tissues

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*Summary.* The influences of dilution, storage and pH on the distribution of 99m-technetium methylene diphosphonate (99m-Tc-MDP) in bone and various soft tissues have been investigated in the rat. The results show a marked departure from normality when large dilution factors and very acidic or basic preparations are used.

The preparation of a stannous methylene diphosphonate kit (Sn-MDP) and its subsequent labelling with 99m-technetium (99m-Tc) has previously been described[1]. This agent is selectively taken up by the hydroxyapatite crystal of bone, and it has been shown to clear very rapidly from blood and soft tissues[1,2]. It is the most suitable of all the bone scanning agents available because of its high in vitro and in vivo stability, coupled with its superior biological properties.

These experiments were conducted in order to investigate the flexibility of use which a lyophilized Sn-MDP kit provides. The effects on bone mineral uptake of dilution,

prolonged storage and pH of the labelled preparation prior to injection have been studied in rats. The results show a departure from normality when large dilution factors and very acidic or basic preparations are used.

*Materials and methods.* 3 groups of experiments were performed in a total of 44 rats. A separate kit was used for each experiment, and the contents of each kit were labelled with 500-800 μCi 99m-Tc in 5 ml volume. Higher levels of 99m-Tc activity were used for the dilution study.

*Dilution.* A 1-ml aliquot of the labelled preparation was dispensed into each of 2 graduated flasks, and diluted by factors of 100 and 1000 respectively. After equilibration at room temperature for 15 min, a dose (20-40 μCi) of each preparation (diluted and undiluted) was injected into the tail vein of 3 separate rats and the animals sacrificed 2 h post injection by an intracardiac overdose of pentobarbitone sodium. A blood sample was taken, and both femurs removed and cleaned of soft tissue components. In addition samples were taken from the following organs for radioassay in a well crystal scintillation counter: liver, kidney, skeletal muscle, thyroid and spleen.

*Storage.* The optimum period for injection of a radiopharmaceutical is usually soon after the labelling had been carried out. In patient studies, however, it may not be possible to inject the material until some hours after labelling as so often happens, when several patients are to be scanned on the same day. To ensure that storage at room temperature does not adversely affect bone uptake and hence scan quality, a dose was dispensed into a syringe, and the rest of the preparation stored in the vial. After storage for 2 h, the dose in the syringe, a dose from the vial, and a freshly prepared dose were injected into 3 separate rats, and the animals killed after 2 h. The same tissues were removed for radioactive counting as in the dilution study.

*pH adjustment.* During the early stages in development of the Sn-MDP kit, localisation studies in animals showed a variation in uptake with respect to bone, liver, spleen and muscle. The unadjusted pH of the preparation ranged from 1.5 to 2.0, and bone uptake was invariably accompanied by a high soft tissue background. Changes in pH values appeared to significantly alter the general pattern of isotope distribution, so it was decided to examine the following pH ranges in animals and by TLC: a) pH = 1.5-2.0; b) pH = 6.4-6.7; c) pH = 9.6-10; d) pH = 13.0-14.0; e) pH = 1.5 → 14.0 → 6.5.

All pH adjustments were carried out using a freshly prepared solution of 6 M sodium hydroxide. A separate rat was injected with each test agent using a similar tracer dose of 20-40 μCi 99m-Tc-MDP and after killing after 2 h, various tissues were removed for radioassay with a sodium iodide counter.

*Results and discussion.* Table 1 shows that a 100-fold dilution results in practically no change in femur uptake of 99m-Tc-MDP, whereas uptake is reduced by half when a dilution factor of 1000 is used. This decrease is probably due to chemical breakdown of the labelled complex which can then lead to one or both of the following: either reoxidation of reduced 99m-Tc(IV) to 99m-Tc(VII) valency state and production of a large amount of free 99m-$TcO_4$, or formation of colloidal particles (technetium and stannous oxides and hydroxides) due to hydrolysis in such a large dilution volume.

An increased soft tissue concentration is noticed particularly in the liver, thyroid and spleen when a dilution factor of 1000 is used. This would suggest presence of both free 99m-$TcO_4$ and colloidal particles. There is little change in bone and soft tissue uptake between the standard preparation and that using 100-fold dilution.

It was decided to examine the effects of dilution on the stability of the preparation because in a previous study[3] an infusion technique was described to demonstrate the variation in extraction of 99m-Tc-labelled ethane-1-hydroxy-1,1-diphosphonate as a function of time. The low concentration of diphosphonate infusion required dilution in a large volume, so pretesting was carried out to determine the optimum dilution factor which will not significantly affect the stability of the preparation.

It is also known that in some technetium generators, a large eluate volume may be necessary towards the end of the generator's life to supply the relatively large nuclide activi-

Table 1. Effect of dilution on uptake of 99m-Tc-MDP in rat tissues (expressed as % dose/g)

| | Standard preparation (undiluted) | 100fold dilution | 1000fold dilution |
|---|---|---|---|
| Femur | 2.28 ±0.32 | 2.01 ±0.41 | 1.16±0.24 |
| Blood | 0.02 ±0.004 | 0.02 ±0.01 | 0.32±0.08 |
| Liver | 0.15 ±0.07 | 0.30 ±0.10 | 0.40±0.18 |
| Kidney | 0.30 ±0.11 | 0.41 ±0.15 | 1.47±0.75 |
| Muscle | 0.004±0.001 | 0.006±0.002 | 0.03±0.01 |
| Thyroid | 0.03 ±0.002 | 0.04 ±0.01 | 2.98±0.97 |
| Spleen | 0.13 ±0.02 | 0.15 ±0.04 | 0.10±0.01 |

Mean of 4 rats ± SD.

Table 2. Effect of storage on 99m-Tc-MDP distribution in rat tissues (expressed as % dose/g)

| | Standard (t = 0 h) | Storage* (t = 2 h) | Storage** (t = 2 h) |
|---|---|---|---|
| Femur | 2.36 ±0.25 | 2.18 ±0.19 | 2.04 ±0.25 |
| Blood | 0.03 ±0.01 | 0.02 ±0.01 | 0.03 ±0.01 |
| Liver | 0.03 ±0.01 | 0.03 ±0.003 | 0.02 ±0.004 |
| Kidney | 0.58 ±0.14 | 0.26 ±0.11 | 0.16 ±0.09 |
| Muscle | 0.002±0.001 | 0.004±0.002 | 0.003±0.001 |
| Thyroid | 0.02 ±0.003 | 0.06 ±0.02 | 0.05 ±0.03 |
| Spleen | 0.02 ±0.01 | 0.04 ±0.01 | 0.06 ±0.02 |

*Stored in syringe. **Stored in labelled vial. Mean of 4 rats ± SD.

Table 3. Distribution of 99m-Tc-MDP in rat* tissues at various pH values (mean of 4 rats ± SD)

| pH | 1.5-2.0 | 6.4-6.7 | 9.6-10.0 | 13.0-14.0 | 1.5→14.0→6.4 |
|---|---|---|---|---|---|
| Femur | 2.07±0.67 | 2.81±0.41 | 2.43±0.58 | 0.11±0.04 | 2.54±0.45 |
| Blood | 0.08±0.02 | 0.03±0.001 | 0.06±0.02 | 0.31±0.10 | 0.84±0.13 |
| Liver | 0.22±0.05 | 0.04±0.01 | 0.21±0.07 | 0.65±0.13 | 0.19±0.05 |
| Kidney | 0.63±0.11 | 0.31±0.09 | 1.06±0.29 | 2.00±0.95 | 0.63±0.11 |
| Muscle | 0.02±0.001 | 0.01±0.002 | 0.01±0.004 | 0.04±0.01 | 0.01±0.002 |
| Thyroid | 0.07±0.01 | 0.04±0.01 | 0.05±0.01 | 8.74±2.05 | 0.06±0.01 |
| Spleen | 0.10±0.003 | 0.03±0.01 | 0.14±0.06 | 0.39±0.10 | 0.08±0.03 |
| TLC** | 93 ±3 | 97 ±2 | 74 ±3 | 16 ±2 | 86 ±3 |

*Expressed as % dose/g tissue. **Expressed as % labelling yield.

ties needed for rapid imaging of the whole skeleton. A large eluate volume may contain a number of oxidants which could lead to a poor labelling yield. It should therefore be realised that any decrease in bone uptake or degradation in image quality of a bone scan might result from such poor labelling of the preparation rather than its instability after the labelling had been carried out.

The results shown in table 2 suggest that there is no significant change in bone or soft tissue uptake between the standard preparation and those which have been stored at room temperature for 2 h before injection. It is a clear advantage to the user in being able to label the diphosphonate some hours before injection as occasional delays in patient arrival and other scheduling problems are sometimes unavoidable. As a delay of up to 4 h between labelling and injection does not affect bone uptake, 99m-Tc-MDP provides great flexibility of use in this respect.

The results of the pH study are summarized in table 3 from which it can be seen that the most marked changes occur at very low and very high pH values. The only variable in these experiments was pH as the weight ratio of MDP to $SnF_2$ was kept constant.

At low pH (1.5–2.0) a low Sn(II) concentration would be desired because of the competition which exists between $Tc^{7+}$ and $Sn^{2+}$ cations for the complexing sites of the MDP molecule. At this pH, $Sn^{2+}$ ions are in excess and will result in some of the 99m-Tc not being able to bind onto the MDP molecule. Low uptake by the thyroid indicates that very little of the added free 99m-$TcO_4^-$ is left in the unreduced state as excess stannous ions should lead to complete reduction.

Labelling efficiency depends on pH, and a high yield is obtained as shown by TLC (93.25±2.71%). This is probably due to the solvent system not being able to separate the bone complex from any hydrolyzed reduced technetium or other colloidal complexes which remain as a single entity at the origin on the TLC strip. A slight increase in liver and spleen activity indicated presence of some colloids.

At high pH (13.0–14.0) a high Sn(II) concentration is desirable for which 2 explanations may be given: either the reduction of 99m-$TcO_4^-$ is rather slow at high pH and a high concentration of the Sn(II) reductant is advantageous, or part of the hydrated technetium oxide is incorporated in a tin hydroxide colloid. At this pH, bone localisation is considerably reduced, and most of the activity appears to reside in the blood and soft tissues. This may account for the high soft tissue concentration seen during the early stages in development of the bone kit and poor labelling yield as shown in the chromatography results (16.11±1.17%).

Other pH values studied (9.6–10.0 and 1.5 → 14.0 → 6.5) showed no change in bone uptake compared to the standard pH value (6.4–6.7) although slight increases occurred in soft tissues. The tissue distribution pattern was consistent with images obtained using a gamma camera. The maximum labelling yield occurs at pH = 6.4–6.7.

These findings indicate that regular monitoring and pH control should be performed on all 99m-Tc-labelled diphosphonate compounds particularly when they are intended for administration into patients such as in routine bone scanning.

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## The influence of hyperosmolality on heart function

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*Summary.* Hyperosmolality produces a reduction in myocardial water content, an increase in ventricular stiffness and coronary blood flow, and a decrease in ventricular performance. The alterations of myocardial dehydration and ventricular stiffness are more pronounced in glucose-induced, and those of ventricular performance in urea-induced hyperosmolality.

Osmolality is an essential physical property of the organism, comparable to temperature in significance. As well as hyperosmolar diabetic coma, diagnostic or clinical treatment with hyperosmolar solutions can account for hyperosmolality of the blood[1–3]. In such cases serum osmolality may rise to, or even above, 400 mOsm/l[1,2]. The effect of glucose- and urea-induced hyperosmolality was, therefore, investigated on heart function.

*Methods.* 32 mongrel dogs of both sexes, weighing 16–26 kg, were anaesthetized with pentobarbital (30 mg/kg) and used to obtain Starling's heart-lung preparations.

The following parameters were measured: cardiac output and coronary blood flow directly (the latter through a cannula tied into the coronary sinus); blood glucose[4], urea nitrogen[5], osmolality (Knauer's semimicroosmometer), haematocrit and oxygen content; left ventricular water content by drying to constant weight; left ventricular pressure by a p23Db Statham gauge through a rigid catheter introduced into the apex and registered on a Hewlett-Packard recorder (Type 1061C); left ventricular passive elastic modulus (characteristic of the diastolic stiffness of ventricular wall) by the modified[6] method of Diamond and Forrester[7].

In order to measure the components of passive elastic modulus and cardiac output over a wide range, the haemodynamic variables were determined at 4 different levels of aortic pressure (40, 80, 120 and 160 mmHg).

After initial determinations, 2 groups of dogs (13–13 animals) were treated either with glucose or urea added to the blood dropwise until the respective doses of 0.5–1.0 and 0.4–0.8 g/kg b.wt was reached. Haemodynamic determinations were made at each stage. The experiment was then concluded in 6 preparations of each group, and the heart was removed for assessment of myocardial water content. In the remaining 7 preparations of both groups, the normal osmolality was reestablished and, after haemodynamic assessment the heart was removed for measurement of myocardial water content. In further 6 preparations, repeated haemodynamic determinations were carried out without altering the blood osmolality, in order to detect the probable occurrence of spontaneous changes. The results were examined statistically using Student's t-test and regression analysis.