PROTEIN LABELLING WITH $^3$H-NSP
(N-SUCCINIMIDYL-[2,3-$^3$H]PROPIONATE)

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SUMMARY
A tritium compound with low molecular weight and diameter, N-succinimidyl-[2,3-$^3$H]-
propionate ($^3$H-NSP), was used to label histones and nonhistone proteins from calf thymus,
and nuclear and total salivary gland proteins from larvae of the midge Chironomus thummi.
Labelled proteins were visualized after electrophoresis in 10% polyacrylamide slab gels by
fluorography. The method is much more sensitive than staining procedures, allowing to
discern approx. 0.1 µg protein.

INTRODUCTION
Protein analysis research of cells and cellular compartments requires reduction of
the quantity of biological material used, as a consequence of the rise of microtech-
niques. This also implies improving conventional (e.g. staining) methods for protein
detection in samples gained by sophisticated micrurgical techniques (Kroeger, 1966;
Lezzi & Robert, 1972; Neuhoff, 1973; Grossbach & Kasch, 1977) which by now aim
at processing of defined eukaryotic chromosomal segments (Bisseling, Berendes &
Lubsen, 1976).

Research has profited from the introduction of ways to label polypeptides to high
specific activities by means of radionuclides of iodine (Bolton & Hunter, 1973;
Rudinger & Ruegg, 1973), a method which was also applied to the study of manually
isolated insect polytene chromosomes and nuclear envelopes (Plagens, 1978).

The use of gamma-emitting radioisotopes of iodine, however, requires special
equipment, laboratory facilities, and high security precautions; it also bears the risk of
generating considerable alterations in protein structure and, mainly due to exposure to
an oxidizing agent such as chloramine T, in protein function (Bolton, 1977). Of course,
coupling of a protein to the molecule described below will to a lesser extent (or even
negligibly) also result in an alteration of protein structure.

Alternatively, there is the possibility of labelling proteins with beta-emitting tritium
compounds which, due to low energy and short range of beta-particles, are efficient
only after enhancing the sensitivity of tritium detection (Randerath, 1970) by adding
a scintillator that converts the energy of $^3$H-particles to light which eventually produce
an image on photographic material (originally proposed by Wilson in 1958).

Boyd, Leach & Milligan (1972) and Leach & Boyd (1973) prepared and studied
N-acylsuccinimides as reagents for the selective acylation of reactive sidechains in
proteins. In short, they found that at pH 6–7 and with molar excesses of acylating
agent of 1.35–6.75 most of the lysine sidechains could be acylated whereas tyrosines reacted only negligibly. The use of N-propionylsuccinimide (pH 7) at molar excesses of 1.35, 2.7, 6.7 and 13.5 over protein amino groups resulted in 84, 100, 100 and 82% lysine modification with only 5, 9, 14 and 52% tyrosine modification (Leach & Boyd, 1973).

At about the same time the 'Bolton & Hunter' reagent, N-succinimidyl-3-(4-hydroxy, 5-[181]iodophenyl)propionate was introduced which yielded protein labellings to high specific activities by reacting with the terminal or sidechain amino groups in proteins to form amide bonds (Bolton, 1977). N-succinimidyl-[2,3-3H]-propionate (3H-NSP) is supposed to react in a similar manner and can be used around or at neutral pH (6–8) as compared to the required alkaline conditions for the 'Bolton & Hunter' reagent.

This paper will describe the application of 3H-NSP to label different proteins for subsequent detection by fluorography.

MATERIALS AND METHODS

Chemicals

N-succinimidyl-[2,3-3H]propionate, sp. act. 46 Ci/mM ≈17TBq/mM (batch 8) was purchased from Amersham-Buchler, Braunschweig, Germany. NSP has a molecular weight of 170 (unlabelled) and its structure is:

$$\begin{align*}
\text{CH}_2^* – \text{CH}_2^* – \text{C} – \text{ON} \\
\text{O} \\
\text{O}
\end{align*}$$

It is supplied in toluene solution and storage is recommended at room temperature where the decomposition is not expected to exceed 1% per month.

Substances for polyacrylamide gel electrophoresis (10% acrylamide/0.27% bis-acrylamide) were as described by Laemmli (1970) and for fluorography instructions of Bonner & Laskey (1974) and Laskey & Mills (1975) were followed.

Calibration proteins were from Boehringer Mannheim GmbH: combithek®, calibration proteins size 1.

Animals

Adult fourth instar larvae of the midge Chironomus thummi were used.

Chromatin

Calf thymus was prepared and kindly provided by Dr N. U. Bosshard; for details of preparations see Bosshard (1979).

Histones

Histones were extracted from calf thymus chromatin; one additional histone (H4) was from Boehringer, Mannheim, also from calf thymus.
**Protein labelling with $^3$H-NSP**

**Salivary glands**

Glands were dissected from adult fourth instar larvae (eL4; staging according to Kroeger, 1973). One to 20 glands were transferred to a piece of parafilm 'M' (American Can Company, Dixie/Marathon, Greenwich, Ct.) and brought into conical centrifuge tubes filled with 50-100 µl denaturing sample buffer (0.0625 M Tris-HCl, pH 6.8; 2 % Na-dodecylsulphate (SDS); 10 % glycerol; 0.001 % bromophenol blue) (Laemmli, 1970). 2-Mercaptoethanol was found unnecessary; it is said to gradually lose some of its potency in solution (LeStourgeon & Beyer, 1977) resulting in smeared bands after electrophoresis. The tubes were sealed with parafilm and heated for 5 min in a boiling waterbath.

**Nuclei**

Nuclei were isolated from salivary glands according to a method developed by Robert (1975) which, briefly, consists of passing Ringer-detergent treated salivary glands repeatedly through narrow capillary pipettes. This finally results in setting free nuclei which were also dissolved in denaturing buffer.

**Chromatin and histones**

Lyophilized chromatin or histone samples (mostly 1 mg/ml) were dissolved in denaturing buffer and diluted to appropriate concentrations.

**Labelling**

The glass vial containing $^3$H-NSP (1 mCi/500 µl) was placed into ice-water to reduce evaporation of the solvent; samples of $^3$H-NSP solution were put into conical centrifuge glass tubes and stoppered with silicone plugs. Inserting 2 syringe needles, the solvent was evaporated in a gentle stream of nitrogen. According to manufacturer's instructions evaporation of 1 ml toluene takes 20 min at 20 °C. Denatured protein samples were then transferred to the glass tubes, stirred vigorously and immersed for reaction in a 37 °C waterbath for 30 min after which they were ready for electrophoresis.

**Electrophoresis**

Buffers and gels (1.5 mm) were prepared according to Laemmli (1970) with a minor modification concerning TEMED which was used in a final concentration of 0.25 %. The gels were run in a Desaga (Heidelberg, Germany) screening electrophoresis separating unit (system Havanna); running buffer was cooled to 15 °C and gels were run for 60-90 min at 70-75 mA/150-220 V. The gels were stained overnight with 0.025 % Coomassie Brilliant Blue G-250 (Serva, Heidelberg) in 50 % methanol/7 % acetic acid and destained in 5 % methanol/7 % acetic acid. They were then ready for densitometry (Kipp & Zonen densitometer DD 2 and flatbed recorded BD 7) and photography.

**Fluorography**

The instructions of Bonner & Laskey (1974) and Laskey & Mills (1975) were followed: the gels were soaked in DMSO for 2 x 30 min, then for 3 h in a 22 % solution of PPO (2,5-diphenyl-oxazole) in DMSO (w/v). PPO was precipitated in situ by immersing the gels in deionized water (60 min) and dried under vacuum with a slab gel drier GSD-4 (Pharmacia, Sweden). They were then brought in tight contact with a preflashed (Laskey & Mills, 1975) Kodak X-Omat (X-ray) film and stored in light-tight boxes at −70 °C for the periods indicated. After developing (Kodak D 19), fixing and drying they were ready for densitometry and photography.

The use of DMSO and PPO can be avoided by (i) a new product from NEN: autoradiographic enhancer EN'HANCE™, or by (ii) the application of a water-soluble fluor, sodium salicylate (Chamberlain, 1979); the latter compound is inexpensive and the method less time-consuming.
RESULTS

Calibration proteins (100 μg/ml each) were labelled with 20 μCi ³H-NSP and after electrophoresis yielded fluorograms which could be scanned densitometrically (not shown).

Chromatin. Fifty microlitres of chromatin (1 mg/ml) in denaturing buffer were labelled with 10, 20, 40 and 60 μCi ³H-NSP, electrophoresed, stained with Coomassie blue and further processed for fluorography. Fig. 1 shows in A the pattern after Coomassie blue staining: only histones are to be seen. Histone H1 is represented by 2 bands, H3 and H2B are not separated in this 10% gel and comigrate in 1 band, H2A and H4 give 1 band each. B and C are both fluorograms after 48 and 140 h exposure, respectively. It is quite striking that many more bands (mostly nonhistone proteins, but also 1 additional H1 band) are resolved; this in turn depends on concentration of label, length of exposure, and quantity applied to the gel: it seems that in C the second run from the right represents an 'optimal' resolution as far as small and faint nonhistone protein bands are concerned.

Salivary glands dissolve completely in buffer containing 2% SDS and, when labelled with, e.g. 20 μCi ³H-NSP, show distinct banding patterns (Fig. 2) which also exceed the details of conventional Coomassie blue staining. In the beginning usually 10 glands were used but I found that one gland will give enough resolution. Grossbach (1977), with a micromodification of the Lowry technique, determined the total protein content of salivary glands of the related species C. tentans to be around 15 μg/gland (mean calculated from Grossbach's data); since the salivary gland of C. thummi is smaller with about 30 cells (40 in C. tentans) I assume a protein content of approx. 10 μg/gland. Hence, application of 10 μl of 1 gland dissolved in 50 μl buffer equals 0.2 μg of salivary gland protein which is visibly resolved.

Nuclei also give a clear banding pattern after ³H-NSP labelling and fluorography (Fig. 3). Here appears the advantage of the method: due to the rather small amount of protein present in nuclei (see below) Coomassie blue staining of the gels reveals only some extremely faint bands (the most prominent histones) in cases where as much as 1000 nuclei had been used. I found that after labelling the amount of protein equivalent to 50–100 nuclei gives reasonable resolution after fluorography. Assuming a ratio of 1:2 for DNA:protein and a DNA content of 3 × 10⁻⁹ g/nucleus (Daneholt & Edström, 1967; Serfling et al. 1975, have reported 1.25 × 10⁻⁹ g/nucleus) then 50–100 nuclei represent 0.3–0.6 (or 0.125–0.25) μg protein which is still above the detection limit of this method. In fact, depending on the ³H-NSP concentration applied, less material may be needed (in preparation).

Fig. 1. Calf thymus chromatin separated in 10% polyacrylamide. A, Coomassie blue stain; B, fluorogram, 48 h exposure; C, fluorogram, 140 h exposure. 1–4, chromatin labelled with 10, 20, 40 or 60 μCi ³H-NSP and 10 μl (± 10 μg chromatin) applied to each slot of the gel. 1'–4': Ditto, but 5 μl applied.
Protein labelling with $^3$H-NSP

A

= H1

H3/2B
H2A
H4

±

B

C
Histones. Total histones from calf thymus (1 µg in 10 µl) were labelled with 5, 10 or 20 µCi ³H-NSP (molar ratios ³H-NSP:protein 1:15, 2:3 and 4:6, assuming a mean mol. wt of 15,000 for histones and using a ³H-NSP batch with a sp. act. of 66 Ci/mM); 0.5, 0.3 and 0.1 µg were run in the gel. Peak areas of H₃/H₂B + H₂B bands were determined after densitometry of the fluorogram (Fig. 4); as little as 0.3 µg total histone can be detected after labelling with only 5 µCi ³H-NSP (= 2.5 µl). Histones H₁ (1 µg/50 µl) and H₄ (1 µg/50 µl or 10 µg/50 µl) were separately labelled with 5, 10 or 20 µCi ³H-NSP (molar ratios for H₁: 2:28, 4:57 or 9:13; for H₄ (1 µg/50 µl):

![Diagram of histone bands and fluorogram](image)

Fig. 2. *Chironomus thummi* salivary gland proteins labelled with 20 µCi ³H-NSP (exposure time 12.5 days). Numerals denote identifiable bands with approximate mol. wt: 1, 104,000; 2, 95,000; 3, 88,000; 4, 80,000; 5, 65,000; 6, 58,000; 7, 50,000; 8, 43,000; 9, 39,000; 10, 34,000; 11, 30,000; 12, 27,000; 13, 25,000; 14, 22,000; 15, 19,000; 16, 18,000; 17, 12,000; 18, 11,000. The fluorogram represents proteins from 3 salivary glands.

1-23, 2-45 or 4-91), 1-5 µg or 0.1-0.5 µg applied to the gel and the fluorograms evaluated after various exposure times (2-28 days) for visibility of bands. It could be shown for histone H₁ that labelling with 10 µCi ³H-NSP and an exposure time of 28 days equals labelling with 20 µCi ³H-NSP and an exposure time of 7 days, i.e. doubling the label reduces the necessary exposure time 4-fold. However, the 'optimal' procedure for 0.1-0.5 µg H₁ seems to be an amount of 20 µCi ³H-NSP and 14 days exposure. Histone H₄ (0.1 µg) labelled with 10 µCi ³H-NSP is not visible even after 28 days exposure, otherwise conditions are comparable to those for histone H₁ despite the extremely different lysine/arginine ratios in the 2 materials. Reducing the molar ratio 10-fold but applying 10 times more protein to the gels gives good results with 10 µCi after 14 days and acceptable results with 20 µCi after 48 h.
DISCUSSION

It is the purpose of this paper to report on the application of a new, low-molecular-weight substance which permits careful labelling of proteins in submicrogramme quantities. As a tritium compound it is intentionally thought to be an alternative to radionuclides of iodine to the extent that facilities for work with these substances are not available or that the procedure with chloramine T risks interference with biological, immunological and receptor-binding activity. The latter difficulty can be overcome by the use of lactoperoxidase iodination (Parsons & Kowal, 1979) or electrolytic procedures (Teare & Rosenberg, 1978; Sammon, Stansbury & Stahr, 1979) with sample sizes $\geq 0.5\, \mu g$ protein.

It could be shown that nonhistone proteins from calf thymus chromatin can be depicted to a certain extent after $^3H$-NSP labelling and subsequent fluorography which until now was unsuccessful with Coomassie blue staining. It will, however,
remain a qualitative procedure which can be outdone by quantitative extraction and separation methods.

Concerning the salivary gland proteins and proteins of isolated nuclei, the main result is that the amount necessary for electrophoretic separation could be lowered considerably; it is intended to separate nuclear proteins of one single gland. Plagens (1978) claimed to be able to analyse the protein complement of 15 polytene genomes within 2 days after iodination with the chloramine T method; it should be interesting to label nuclei in parallel experiments with iodine and NSP and to compare the resulting patterns. The $^3$H-NSP method established here was, however, not intended for optimization in micro-scales nor for speed in obtaining results, but rather for less exigent routine work.

A future goal, comparing proteins of single nuclei, can only be achieved with the aid of micromethods (Neuhoff, 1973); it seems to have been approached by demonstration of the separation of proteins from as little as 10 salivary gland nuclei by means of micromanipulator-assisted microelectrophoresis with gels 50–100 $\mu$m in diameter (Grossbach & Kasch, 1977).

Another tritium-labelled compound, $^3$H-dansyl chloride (DCI), originally and still used in amino acid analysis, was recently proposed for the quantitation of proteins (Schultz & Wassarman, 1977). Unfortunately, the sensitivity of the $^3$H-DCI assay is reduced 100-fold in the presence of nucleophilic compounds (e.g. dithiothreitol or mercaptoethanol) so in cases where these compounds are indispensable $^3$H-DCI will be of no use.
Protein labelling with \(^3\)H-NSP

Recently, a different and promising method for tritium labelling of submicrogramme quantities of protein (down to around 10 ng) was proposed: proteins are labelled \textit{in vitro} by reductive methylation of amino groups with formaldehyde and high specific \(^3\)H-potassium borohydride (\(^3\)H-KBH\(_4\)) (Kumarasamy & Symons, 1979). After SDS-polyacrylamide gel electrophoresis and fluorography protein bands can be evaluated in a manner similar to the one described here.

Comparable results should also be achieved by labelling proteins prior to electrophoresis with fluorescent reagents: e.g. fluorescamine (Fluram) (Castell, Pestana, Castro & Marco, 1978; Ragland \textit{et al.} 1978; Sung, Bozzola & Richards, 1978) or MDPF (2-methoxy-2,4-diphenyl-3(2H)-furanone) (Goldberg & Fuller, 1978; Douglass, LaMarca & Mets, 1978). A detection limit of 1 ng is claimed for the use of MDPF requiring, however, instrumentation for fluorescence gel scanning and extreme precautions against fluorescent contaminants of glassware.

The use of mercaptoethanol in the \(^3\)H-NSP assay does not interfere with the reaction (not shown) but further tests will have to be carried out to determine the specificity of the reaction and possible interferences with cellular macromolecules.

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REFERENCES


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