N-succinimidyl propionate: Characterisation and Optimum Conditions for use as a Tritium Labelling Reagent for Proteins

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SUMMARY

N-Succinimidyl propionate [NSP] was synthesised and characterised as a pale yellow waxy solid. Rates of hydrolysis in buffers range pH 5-9 were determined. Optimum conditions for reaction with lysine were within the range pH 7 to 8. A protein, wheat germ agglutinin was labelled at pH 7.5 at room temperature for two hours. Rates of reaction of other substrates under these conditions were investigated.

Key Words: Protein labelling, tritium, kinetics.

1. INTRODUCTION

N-Succinimidyl [2,3-^{3}H]propionate (NSP) is now a well established reagent for the introduction of a tritium label into proteins. This reagent provides a simple method for the labelling and detection of sub-microgram quantities of proteins.

However, the unlabelled compound which is required by users of the tritium labelled material for certain calibration experiments has never been properly characterised. The reagent has been assumed to react with the epsilon amino group of lysine in proteins as shown in equation 1.

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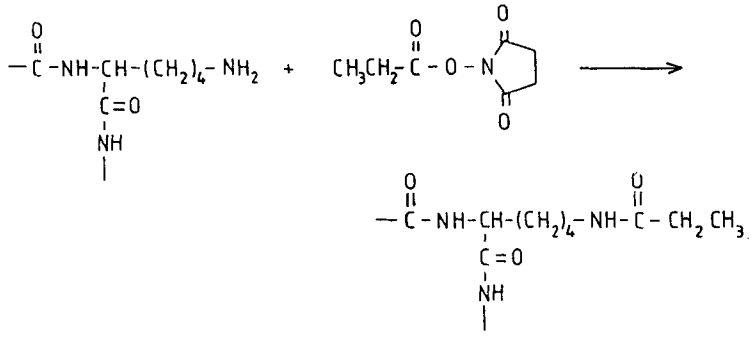

Already a number of papers have appeared which demonstrate the utility of the reagent. For example, the retrograde transport of protein components of sciatic nerve axons has been demonstrated by direct labelling of the protein using [^{3}H]NSP *in vivo*. In this new approach to the investigation of axonal transport by Fink and Gainer [1], it was shown that NSP had no adverse effect on cellular function. Similarly, Oakleford and Clint [2] reported the preparation of [^{3}H]IgG for their studies into placenta uptake during pregnancy. Muller [3] described the use of NSP to label histones and non-histone proteins from calf thymus and nuclear and total salivary gland proteins from the larvae of the midge *Chironomus thummi*. The labelled proteins were visualised by fluorography after electrophoresis in polyacrylamide gels. Dolly et al [4] have shown that α-bungarotoxin labelled with [^{3}H]NSP and purified by electrofocusing retained its biological activity. Supernatant Protein Factor (SPF) has been labelled by Caras, Friedlander and Block [5]. Kummer et al [6] have reported recently the labelling of very large numbers of immunoglobulins with [^{3}H]NSP. They found the labelled conjugates to be suitable for the detection of activity in hybridoma supernatants and demonstrated that there was no loss of functional activity.

In this paper we describe the synthesis, the purification by crystallisation, and the characterisation of NSP, and also the hydrolysis rates of NSP over the pH range 5-9 in various buffers at 20°C. Kinetic experiments to establish the optimum reaction conditions with lysine and wheatgerm agglutinin are described using N-succinimidyl[2,3-^{3}H]propionate. The results of the experiments using a number of substrates under these optimum conditions are presented.

2. EXPERIMENTAL

2.1 Preparation of N-succinimidyl propionate

Ethyl acetate (250 ml) was dried by standing over molecular sieve (type 4A) for 24 hours. N-Hydroxysuccinimide (3.2g, 2.8 mmol) was dissolved in ethyl acetate (120 ml). Propionic acid (2 ml, 28 mmol) followed by dicyclohexylcarbodiimide (5.8g, 28 mmol) in dry ethyl acetate was added. The mixture was stirred overnight. The precipitate of dicyclohexylurea was removed by filtration and washed with ethyl acetate (10 ml). The solution was rotary evaporated to small volume and left at room temperature for about two hours, and the small precipitate of dicyclohexylurea was again filtered off. The solution was rotary evaporated to "dryness" and redissolved in ethyl acetate (0.8 ml). Hexane (1.2 ml) was added dropwise until opalescence occurred. The mixture was stored at -20°C. Crystals were formed overnight and filtered using a Millipore (type 3M) filter, and dried at room temperature under vacuum to give a pale yellow waxy solid (2.5g). The crystals were recrystallised from ethyl acetate:hexane. Extensive drying under vacuum at room

temperature failed to remove the last traces of ethyl acetate apparent as signals at 4.12, 2.03 and 1.25 as shown by the pmr spectrum.

0.79,t,3H, $CH_2\underline{CH_3}$; 1.68,m,2H, $\underline{CH_2}CH_3$;

2.10,q,4H,$CO\underline{CH_2CH_2}CO$.

An analytical sample was further purified, by exploiting the low volatility of NSP, by sublimation onto a cold finger under vacuum.

mp, 32-34°C

electron impact m/e 15, 28, 29, 57, 98, 115, 156, 171, 172,

i.r. 1735, 1780, 1820 cm^{-1}

Microanalysis Calculated	%C:H:N:O	49.12 : 5.30 : 8.18 : 37.39
Found	%C:H:N:O	49.33 : 5.17 : 8.48 : 37.03

2.2 Kinetic Experiments

N-Succinimidyl[2,3-^{3}H]propionate (TRK 556) (7.5 mCi, 50 Ci/mmol, Amersham International plc) as a solution in toluene was carrier diluted with a toluene solution of N-succinimidyl propionate (0.12mg). The mixture was rotary evaporated just to dryness. To this residue, buffer or buffer plus substrate was added (1 ml) and swirled intermittently at the required temperature. The solution was sampled (5µl) at regular time intervals onto silica gel G tlc plates (without fluorescent indicator). The plates were eluted without drying with petroleum ether (60°C - 80°C):ethyl acetate (70:30). The plate was sprayed with 10% sodium hydroxide in methanol before drying to fix the hydrolysis product prior to scanning. The time between completion of elution and scanning was kept constant. The ratio of [^{3}H]NSP (Rf = 0.35) to the hydrolysis products was calculated by

triangulation. The percentage decrease in [^{3}H]NSP against time was plotted and the rates of reaction were obtained from the gradients of the curves expressed as percentage change per minute.

Reactions at pH >9 were not carried out because under these conditions hydrolysis was proceeding so rapidly that meaningful assays could not be conducted.

Table 1 Results of reaction between N-succinimidyl propionate and buffer, or buffer with substrates

pH (Buffer) at 20°C		Percentage rate of reaction min^{-1}		
		No additive (hydrolysis)	With lysine(1)	With wheat germ(2) agglutinin
5	(sodium acetate)	0.075	0.18	0.16
6	(0.2M phosphate)	0.16	0.75	0.45
7	(0.2M phosphate)	0.62	3.22	1.30 (0.57)*
7.5	(0.2M phosphate)	0.84	5.33	1.80
8	(0.2M phosphate)	1.00	6.00	3.10 (1.47)*
8.1	(borate)	1.18	6.50	2.8
9	(borate)	8.80 (decomposition)	-	-

* Experiments at 0°C

(1) concentration 0.8µmol/ml

(2) concentration 5mg/ml

3. RESULTS AND DISCUSSION

The rate of hydrolysis of N-succinimidyl propionate increased with pH and above pH 8.0 degradation products other than propionic acid were observed. The half life of NSP was 6.6 min at pH 9.0 and 660 min at pH 5.0. Yet faster rates were observed in the presence of various substrates. This data is shown in table 1.

The reaction rate with wheat germ agglutinin was much slower than that for lysine, presumably due to steric hindrance. Temperature had the anticipated effect in reducing the rate by a factor of two between 20°C and 0°C.

Since most proteins are more stable within the range pH 4.0-8.0 and are usually stable for short periods in time in solution at room temperature optimum conditions were concluded to be pH 7.5 buffer at room temperature for approximately two hours. Subsequent experiments using different substrates were carried out under these conditions and the results are shown in Table 2.

From Table 2 it may be seen that the sulphydryl-SH functional group is very reactive while a disulphide bridge in a peptide is unlikely to be affected during the course of reaction. Lysine is more reactive than other amino acids and its unhindered epsilon amino group reacts at approximately twice the rate of the alpha amino group. Histidine has a substantial reaction rate with NSP under these conditions therefore labelling of this group is likely in proteins. Reaction rates of NSP with arginine, tryptophan and phenylalanine are significant, so N-terminal labelling of peptides is possible provided the N-terminal is not sterically hindered.

NSP has been used in vivo [1] and reaction with adenosine and cytosine demonstrate that reaction with polynucleotides would be minimal. As expected NSP does not react with neutral carbohydrates.

Table 2 Results of reaction between NSP and substrates in phosphate buffer pH 7.5, 20°C for 2 hours

Substrates	Percentage rate of reaction min^{-1}	Corrected* Reaction rate with substrate min^{-1}
none	0.84	-
2-Mercaptoethanol	11.43	10.59
Lysine	5.33	4.49
N-α-t-Boc-L-histidine	2.93	2.09
N- -t-Boc-L-lysine	2.296	1.45
Wheat germ agglutinin	1.80	0.96
N-α-t-Boc-L-arginine	1.79	0.95
DL-phenylalanine	1.63	0.79
N-t-Boc-L-tryptophan	1.49	0.65
Adenosine	1.39	0.55
Cytosine	0.96	0.12
2-Hydroxyethyldisulphide	0.84	-
D-glucose	0.82	-

* Percentage reaction rate minus rate of hydrolysis

4. CONCLUSION

NSP reacts well with primary amines. Proteins may be labelled with N-succinimidyl-[2,3-^{3}H]propionate in the range pH 7.5-8.0 at room temperature for 1 to 2 hours or for longer periods at 0°C. This reaction time will accommodate the lower reaction rate of lysine groups which to some extent will be sterically hindered when incorporated in proteins. When present, cysteine residues will react with NSP. Thiol reagents, which are often used to stabilise some proteins, must be absent when labelling with N-succinimidyl-[2,3-^{3}H]propionate.

References:

1. Fink D.J. and Gainer H. - Science. 208: 303-308 (1980)

2. Ockleford C.D. and Clint J.M. - Placenta, 1: 91-111 (1980)

3. Muller G.H. Journal of Cellular Science, 43: 319-328 (1980)

4. Dolly J.O. Nockles E.A.V., Lo M.M.S. and Barnard E.A. - Biochemical Journal, 193: 919-923 (1981)

5. Caras I.W., Friedlander E.J. and Bloch K. - Journal of Biological Chemistry, 255: 3575-3580 (1980)

6. Kummer U., Doxiadis I., Thiel E., Eulitz M. and Thierfelder S. - Journal of Immunological Methods, 42: 367-374 (1981)

7. McLean W.G. and Meiri K.F. - Journal of Physiology, 317: 82 (1981)