Glycoconjugate J (1986) 3: 153—161

CMP-*N*-Acetylneuraminic Acid: Is it Synthesized in the Nucleus?

WIJNHOLT FERWERDA*, CORRIE M BLOK and JOHANNA VAN RINSUM[1]

Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, NL-1007 MC Amsterdam, The Netherlands
[1]*Present address: Division of Cell Biology, Lab. Exp. Cytology, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands*

Received November 18, 1985/February 27, 1986.

Key words: CMP-N-acetylneuraminic acid, sialic acid, nucleus

Radioactive *N*-acetylmannosamine was injected intravenously into rats to label *N*-acetylneuraminic acid (NeuAc) and CMP-NeuAc. Nuclei were isolated from the livers using a non-aqueous technique to prevent leakage of polar metabolites. A preparation was obtained, which was eight times enriched in nuclei based on the ratio DNA/RNA. Free NeuAc and CMP-NeuAc were isolated from this nuclear fraction and from whole liver, and the specific radioactivities were determined. It appeared that at all time points studied, i.e. 1.5, 9.5, and 18 min after injection, the specific radioactivities of free NeuAc as well as of CMP-NeuAc in the nuclear preparation were lower than those in whole liver. Also no significant differences were found between free NeuAc and CMP-NeuAc in the ratio of specific radioactivities in the nuclear fraction/whole liver. Furthermore, no enzyme involved in the synthesis of NeuAc was enriched in the nuclear preparation as compared to various other cytosolic and non-cytosolic enzymes.

Because newly synthesized NeuAc is channelled into a special pool and used for activation to CMP-NeuAc [Ferwerda W, Blok CM, van Rinsum J (1983) Biochem J 216:87-92], these results point to a site of activation of NeuAc to CMP-NeuAc other than the nuclear compartment. This might indicate that the nuclear-localized enzyme, CMP-NeuAc synthase, leaves the nucleus before exerting its action.

Abbreviations. ManNAc kinase (EC 2.7.1.60), ATP:2-acetamido-2-deoxy-D-mannose 6-phosphotransferase; GlcNAc kinase (EC 2.7.1.59), ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase; NeuAc 9-phosphatase (EC 3.1.3.29), *N*-acetylneuraminate 9-phosphate phosphohydrolase; CMP-NeuAc synthase (EC 2.7.7.43), CTP:*N*-acetylneuraminic acid cytidylyltransferase; glucose 6-phosphatase (EC 3.1.3.9), D-glucose 6-phosphate phosphohydrolase; *p*-nitrophenylphosphatase (EC 3.1.3.1/2), orthophosphoric monoester phosphohydrolase; LDH (EC 1.1.1.27), L-lactate:NAD oxidoreductase; β(1-4)-galactosyltransferase (EC 2.4.1.38), β-*N*-acetylglucosaminide β(1-4)-galactosyltransferase.

*Author for correspondence

Sialic acids, the negatively charged terminal sugars of many glycoconjugates, are synthesized as *N*-acetylneuraminic acid (NeuAc) in the cytosol [1, 2]. The NeuAc-activating enzyme, CMP-NeuAc synthase, has been found in the nucleus [3-9], in contrast to other sugar-activating enzymes which are found in the cytosol [1, 8]. The physiological significance of this specific localization of the CMP-NeuAc synthase is still unknown, but it might play a regulatory role in the biosynthesis and transfer of sialic acid.

Recently, we were able to show that newly synthesized NeuAc molecules in the cell are channelled into a special pool and are preferentially used by the enzyme CMP-NeuAc synthase. This conclusion was based on the observation that, after injection of [^{3}H] ManNAc into the rat, the specific radioactivity of CMP-NeuAc became higher than that of free NeuAc. This phenomenon was observed in brain [10], liver [11] and kidney [12]. Because of the nuclear localization of CMP-NeuAc synthase, we assumed the special compartment to be the nucleus. We were interested in the interrelationship between the cytosol-localized NeuAc-synthesizing enzymes and the nuclear-localized CMP-NeuAc synthase, and in addition, how the product, CMP-NeuAc is transported to the Golgi vesicles, where it acts as NeuAc donor in sialyltransferase reactions. In an attempt to study this problem, we first wanted to prove that the synthase is active in the nucleus *in vivo*. Therefore, we labelled NeuAc and CMP-NeuAc in rat liver by i.v. injection of [^{3}H] ManNAc, and after some time the nuclei were isolated in organic solution to determine the specific radioactivities of NeuAc and of CMP-NeuAc in the nuclear compartment.

Contrary to our expectations, we were not able to obtain any evidence for the synthesis of CMP-NeuAc in the nucleus *in vivo*. In the present paper we report the results of our experiments. Part of this work has been presented in a preliminary form [13].

Materials and Methods

Materials

N-Acetyl-D-[6-^{3}H(N)]mannosamine (19 400 Ci/mol), *N*-acetyl-[4-^{14}C]neuraminic acid (4 Ci/mol), CMP-*N*-acetyl-[4-^{14}C]neuraminic acid (1-2 Ci/mol) and *N*-acetyl-D-[1-^{14}C]glucosamine (58.2 Ci/mol), were obtained from New England Nuclear, Boston, MA, USA. *N*-Acetyl-D-[U-^{14}C]mannosamine (254 Ci/mol) and UDP-[^{14}C]galactose (340 Ci/mol) were purchased from Amersham International, Amersham, UK. For the *in vitro* incubations, labelled substrate was diluted with unlabelled substrate to lower the specific radioactivities. [^{14}C]ManNAc 6-phosphate (0.1 Ci/mol) and [^{14}C]NeuAc 9-phosphate (0.05 Ci/mol) were prepared from [^{14}C]-ManNAc as described earlier [2]. Dowex AG 1-X8 (100-200 mesh; Cl^- form) was obtained from Bio-Rad, Richmond, CA, USA and was converted into the HCO_3^- form. Cyclohexane and carbon tetrachloride were from Merck, Darmstadt, W. Germany, and were dried with $CaCl_2$ before use. All other chemicals were obtained from commercial sources and were of the highest quality.

Animals

Male Wistar rats (2-3 months old, 190-220 g) were kept on a light-dark schedule and fed two hours a day as described earlier [11]. On the day of injection no food was given.

Two series of rats, each containing seven or eight rats, were injected intravenously with 0.5 ml of phosphate-buffered saline (10 mM sodium phosphate, 0.154 M NaCl), pH 7.4, containing 100 μCi of [^{3}H]ManNAc, 1.5 min and 18 min before killing. Another series of rats was injected 9.5 min before killing with the same solution containing 10 μCi of [^{14}C]ManNAc. These rats had been injected first subcutaneously with 100 μCi of [^{3}H]ManNAc (double labelling experiment) 2.5 h before killing. For the determination of the nuclear enzyme activities one series of four rats was used without prior injection of radioactivity. Rats were killed by immersing the whole animals in liquid N_2. The livers were removed in a frozen state [11] and immediately lyophilized on a cold plate at -15°C. The dried material was ground in a mortar in a plastic bag and subsequently pressed through a nylon sieve (210 nm). The material was stored until use at 0°C under vacuum in a desiccator containing P_2O_5.

Isolation of Nuclei

Nuclei were isolated by a modification of the method described by Siebert [14]. A mixture of dried cyclohexane and carbon tetrachloride (65/36 by vol) was cooled at -4°C and the density, 1.32-1.33 g/ml, was checked by weighing. Approximately 600 mg of the powdered liver were mixed with 20 ml of the cooled organic mixture and homogenized under cooling in ice with a motor-driven glass/Teflon Potter-Elvehjem homogenizer (clearance 0.080 mm), five strokes up and down, 2 850 rpm.

The homogenate was centrifuged in a swing out rotor in a centrifuge equipped with a closed rotating case, in the cold (0°C) at 2 000 × *g* for 60 min. After centrifugation the temperature had increased a few degrees, and as a result the density dropped to approx. 1.315-1.325 g/ml. A small pellet was obtained enriched in nuclei, separated by an almost clear middle layer from a large floating layer. The pellet was carefully withdrawn together with some middle layer with a Pasteur pipette. This operation always caused mixing of the floating layer with the middle layer. Therefore, the pellets of four tubes were combined and centrifuged again at 2 000 × *g* for 60 min. The small floating layer with the organic solution was withdrawn and the pellet was dried in a microrotavapor (pellet 1). The floating layer of the first centrifugation step was mixed with the remaining middle layer and adjusted to the original volume with the organic mixture. Following the same centrifugation procedure a second pellet was obtained. The dried pellets were stored until use at 0°C in a desiccator containing P_2O_5 under vacuum. For analysis, the organic middle layer and the floating layer were also sampled, dried and stored in a desiccator. Samples from all fractions and from dried homogenate were analysed for protein, DNA, RNA, K^+ and Na^+ after suspension in 5 mM Hepes buffer, pH 7.5.

Determination of the Specific Radioactivities of Free NeuAc and of CMP-NeuAc

NeuAc and CMP-NeuAc were purified from powdered liver tissue (total liver) and from the isolated nuclei, and the specific radioactivities (corrected by HPLC analysis) were determined as described earlier [11]. If only [^{3}H]ManNAc had been injected, [^{14}C]NeuAc and CMP-[^{14}C]NeuAc were routinely added as internal standards. Of the total liver, 1 g of powdered tissue was homogenized in 30 ml of cold 75% (v/v) ethanol.

All nuclei obtained from one series of rats, which met the criterion of purity (relative ratio DNA:RNA >6) were combined, being 7-8 mg of DNA totally, and extracted with 30

ml of cold 75% (v/v) ethanol. The total quantity of NeuAc and of CMP-NeuAc at the end of the purification procedure obtained from the combined nuclei of one series was sufficient for two micro-determinations with the accessory countings and HPLC analyses.

Electron Microscopy

A sample of dried nuclear pellet was suspended in 0.25 M sucrose, containing 10 mM Hepes buffer pH 7.5. After 30 min at 4°C the nuclei were spun down in an Eppendorf centrifuge at 14 000 rpm and fixed in the same buffered sucrose solution containing 1.5% glutaraldehyde. The nuclei were again centrifuged and postfixed in a sucrose solution containing 1% OsO_4, dehydrated, left to stand overnight in Epon-Araldite/Propylene oxide (1/1 by vol) and embedded in Epon-Araldite. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate.

Enzyme Assays

To determine the activities of various enzymes in the dried nuclear fraction and the homogenate, samples were suspended in 0.25 M sucrose, containing 10 mM Hepes buffer, pH 7.5. To remove the outer nuclear membrane, including adsorbed cytoplasmic material [7], Triton X-100 was added to a part of the nuclear suspension to a final concentration of 1% and incubated for 30 min at 0°C. The suspension was centrifuged at 1 000 × *g* for 5 min and the pellet was resuspended in buffered sucrose. GlcNAc kinase, ManNAc kinase, CMP-NeuAc synthase, NeuAc 9-phosphatase, glucose 6-phosphatase (specific; measured under optimal conditions: unspecific; measured under conditions of NeuAc 9-phosphatase assay), and *p*-nitrophenylphosphatase (unspecific) were determined as described earlier [1, 2]. LDH was determined according to the method of Lisman *et al.* [15] and β(1-4)-galactosyltransferase as described by Blanken *et al.* [16] with *N*-acetylglucosamine as acceptor.

Analytical Methods

DNA and RNA were extracted from the samples by heating at 70°C for 20 min in 1 M $HClO_4$ [17]. DNA was determined with diphenylamine [18] and RNA with orcinol [19]. Na^+ and K^+ were determined by flame-ionization. Protein was measured by the method of Lowry *et al.* [20] with bovine serum albumin as standard. Sialic acid was determined by the thiobarbituric acid method [21] on a microscale suitable for quantities of 1 nmole per determination. Radioactivity was counted in a Berthold liquid scintillation counter (model BF 8 000) with automatic calculation of dpm.

Results and Discussion

Studies concerning the compartmentalisation of low molecular weight metabolites are hampered by the fact that redistribution easily occurs during the isolation procedure. For polar metabolites this problem can be overcome in using non-aqueous media. This technique has been successfully applied for metabolite assays in nuclei [14], in chloroplasts [22], and in mitochondria [23]. For our purpose we needed relatively large quantities of nuclear material, since we had to isolate CMP-NeuAc and free NeuAc from it

Table 1. Composition of and some enzyme activities in a preparation of rat liver nuclei.

Nuclei were purified in non-aqueous solution from the livers of four rats. For the isolation procedure see Materials and Methods. A homogenate of lyophilized liver in organic solution which was left to stand for 2 h at 0°C was used as reference. Dried nuclei or samples from the homogenate dried before use were resuspended in 0.25 M sucrose, containing 10 mM Hepes buffer, pH 7.5. The enzyme assays were carried out in duplicate with the nuclear preparation or the homogenate, containing approx. 0.05 mg or 0.01 mg of DNA, respectively. Non-extractable activity or protein was measured in the pellet obtained after centrifugation of a nuclear suspension which was incubated for 30 min at 0°C with 1% Triton X-100. The yield of this preparation was 9.7% based on DNA. The specific enzyme activities expressed as nmoles/min/mg protein are given in parentheses.

	% recovered relative to DNA	% not extractable from nuclei with TrX-100
DNA	100	N.D.[a]
RNA	12	N.D.
protein	11	21
K^+	12	N.D.
Na^+	33	N.D.
GlcNAc kinase	30 (2.6)	0
ManNAc kinase	6 (0.3)	0
NeuAc 9-phosphatase	3 (0.3)	0
CMP-NeuAc synthase	50 (6.2)	82
Galactosyltransferase	10 (0.15×10^3)	9
Glc 6-phosphatase (unspecific)[b]	4 (1.3)	10
p-Nitrophenylphosphatase	10 (7.1)	0
LDH	6 (598)	0

[a] N.D., not determined.

[b] Glc 6-phosphatase measured under optimal conditions (specific) was 0 in the nuclear preparations.

and to determined the specific radioactivities of these metabolites. Therefore, we developed a two-step procedure based on the method of Siebert [14], giving an acceptable yield in a fast way. The overall yield of our procedure was about 10% based on DNA measurements. The purification factor obtained for the pellets, based on the ratio DNA/RNA varied from 6 to 12. The second pellet was always less pure than the first pellet. The average purification factor of the combined nuclei of the series varied between 8 and 11, a normal value for isolation in non-aqueous media. The purification factor based on the ratio DNA/protein was in the same range. The values of one series (not injected) are shown in Table 1. Our method of freezing was based on the observation that cyclic AMP and cyclic GMP levels in rat tissues were altered by decapitation or anaesthesia and that the best results were obtained by immersing the whole animal in liquid N_2 [24]. This method gave also a higher ATP/ADP ratio for rat liver (0.8 - 1.0) compared to decapitation (0.4 - 0.5, unpublished results). Consequently, it was unavoidable that several seconds elapsed before the inner parts of the liver were deep frozen.

Electron microscopic observation revealed nuclei, most intact, with some adherent subcellular material (results not shown). This was expected since our purification procedure resulted in a preparation enriched approximately eight-fold in nuclei, but which still contained non-nuclear material. During the procedure some nuclei are broken. However, in the organic solution, polar metabolites remain adsorbed to the insoluble material. This was checked by measuring the radioactivity in the organic layer after

Table 2. Specific radioactivities of free NeuAc and of CMP-NeuAc from total rat liver and from rat liver nuclei.

For each time point a series of seven to eight rats were injected i.v. with [^{3}H]ManNAc or [^{14}C]ManNAc. The rats were killed by immersing the whole animals in liquid N_2. Nuclei were isolated in organic solution (for details see Materials and Methods section). Free NeuAc and CMP-NeuAc were isolated and purified from the combined nuclei and from total liver and the specific radioactivities were determined. The values obtained with [^{14}C]ManNAc (9.5 min) were corrected for comparison with the ^{3}H-values (see text).

	Time of injection before killing (min)	Specific radioactivities (dpm/nmol)		Ratio nucl.prep./ total liver
		total liver	nuclear preparation	
NeuAc	1.5	1630	1190	0.73
CMP-NeuAc	1.5	300	230	0.77
NeuAc	9.5	2580	1850	0.72
CMP-NeuAc	9.5	3080	2500	0.81
NeuAc	18	2090	1145	0.55
CMP-NeuAc	18	4060	2195	0.54

homogenization, which was negligible. The nuclei in the floating layer showed the same morphology as those in the pellet.

From the combined nuclei of each series free NeuAc and CMP-NeuAc were extracted and purified and the specific radioactivities were determined. As references the specific radioactivities of free NeuAc and CMP-NeuAc isolated from whole tissue, starting with dried liver, were determined. The results are shown in Table 2. The values obtained with [^{14}C]ManNAc, 9.5 min after injection, were multiplied by 10 for comparison with the [^{3}H]-values (100 μCi [^{3}H]ManNAc versus 10 μCi [^{14}C]ManNAc injected; the percentage absorption of [^{3}H]ManNAc and of [^{14}C]ManNAc by the liver appeared to be in the same range). The striking results of these experiments were the low values obtained for the nuclear fraction. Because newly synthesized Neu Ac molecules are preferentially used for activation [11], the specific radioactivity of NeuAc in the compartment of activation should be high shortly after the pulse, and consequently the specific radioactivity of the resulting CMP-NeuAc should also be high. So, when the specific radioactivity in the whole liver increases (average of all compartments), the specific radioactivity in the compartment of activation will be higher than the average. However, at none of the time points, not even during sharp increases of the average specific radioactivity, was a higher value found in the nuclear fraction. In addition, for each time point no significant differences in the ratio nuclear fraction/whole liver were found between free NeuAc and CMP-NeuAc (see last column of Table 2).

In the double labelling experiments the rats killed 9.5 min after injection of [^{14}C] ManNAc had also been injected subcutaneously with 100 μCi of [^{3}H]ManNAc 2.5 h before killing. We had first determined the course of the specific radioactivities of free [^{3}H]NeuAc and of CMP-[^{3}H]NeuAc versus time in whole liver after subcutaneous injection. It appeared that a slow decline in specific radioactivities occurred between 2 and 4 h; for [^{3}H] NeuAc from about 200 to 150, and for CMP-[^{3}H] NeuAc from about 450 to 200. We concluded that the specific radioactivities of free [^{3}H]NeuAc and of CMP-[^{3}H]NeuAc

in the various compartments did not differ significantly and consequently could be used as reference values. This gave us the opportunity to use the $^{14}C/^{3}H$ ratio as a measure of the incorporation rate of ^{14}C. For whole liver we found a ratio of 0.49 and 0.27 and for the nuclear fraction 0.42 and 0.28 for free NeuAc and CMP-NeuAc, respectively. So this method of determination also resulted in relatively low values for the nuclear fraction, supporting the previous conclusion.

The nuclear preparations appeared to contain about 3.5 nmoles of free NeuAc and 1.8 nmoles of CMP-NeuAc per mg DNA. These values were obtained after correction for the losses during the purification procedure. We found an average of 2.4 mg of DNA per g wet weight, while the volume of nuclei in rat liver can be assumed to be 8% [25]. From these data a nuclear concentration of 105 and 54 nmol/ml (1 ml = 1 g wet weight) can be calculated for NeuAc and CMP-NeuAc, respectively. These values are slightly higher than was found for whole liver [11]. However, if a correction could be made for extranuclear material, these values should become lower. Thus, NeuAc and/or CMP-NeuAc are not stored in the nucleus.

Using a second approach we tried to investigate the function of the nucleus in the activation of NeuAc into CMP-NeuAc. To channel the newly synthesized NeuAc into or on the nucleus, the last enzyme of the synthesis chain, or maybe an enzyme complex, might be adsorbed on the nuclear membrane, delivering the newly synthesized NeuAc directly into a special pool. We therefore isolated nuclei in organic solvent without previously injecting the rats with a radioactive precursor and we measured enzyme activities of the various enzymes involved in NeuAc biosynthesis. As a reference we used dried liver powder, which had been homogenized in organic solution.

First we determined the effect of lyophilizing and of the organic solvent on the enzyme activities. For this purpose homogenates were prepared in 0.25 M sucrose containing 10 mM Hepes buffer, pH 7.5, directly from dried liver powder and from powder first suspended in organic solution for 2 h and re-dried. The enzyme activities were reduced to 20-50% of the original value during the process of freeze drying, but during the organic solvent procedure most enzyme activities were not affected. UDP-GlcNAc 2-epimerase was almost completely denatured, while UDP-GlcNAc pyrophosphorylase and NeuAc 9-phosphate synthase activities became too low for reliable determination. The results obtained for the other enzymes are shown in Table 1. None of the detectable enzymes directly involved in NeuAc biosynthesis was enriched in the nuclear fraction. Several reference enzymes are found in the same concentration range as was the cytosolic enzyme LDH. The bulk of enzyme activities was always found in the floating layer (results not shown), which was analysed to check the recoveries. Of the cytosolic enzymes analysed, only GlcNAc kinase was slightly enriched in the nuclear fraction, relative to protein. After treatment with Triton X-100, all adsorbed enzymes were almost completely solubilized (Table 1), indicating that the enzymes were loosely bound. The residual activities of phosphatases and galactosyltransferase might be due to enzymes which are integral parts of the nuclear compartment [26-28].

The nuclear-localized enzyme, CMP-NeuAc synthase, is found in the highest concentration in the nuclear preparation, although denaturation had occurred because the percentage recovery was only half of that of DNA. This enzyme was not solubilized after treatment with Triton X-100, which is in agreement with the observations reported by

other investigators [6-8]. This results confirms the idea that CMP-NeuAc synthase is a real nuclear enzyme and its localization is not an artefact caused by a redistribution during homogenization in sucrose medium.

Thus, with both approaches we could not obtain any indication that the nucleus has a function in the activation of NeuAc to CMP-NeuAc. It must be realized that our conclusion is based on the assumption that newly synthesized NeuAc and CMP-NeuAc molecules are, at least for a short time, concentrated around the active nuclear CMP-NeuAc synthase. One can postulate that the newly formed CMP-NeuAc is not stored in or on the nucleus, not even for a short time, but that it is returned immediately after synthesis to the cytoplasm ("tennis-ball" effect). In such a case a higher specific radioactivity of CMP-NeuAc in the nuclear compartment will not be found. Earlier we found a lag phase of about 1 min before radioactivity was incorporated into CMP-NeuAc [11]. During this time, a pool of newly synthesized NeuAc is formed with a high specific radioactivity. This pool is preferentially used for CMP-NeuAc synthesis. If this pool is not situated near the site of activation, but is channelled rapidly to the nucleus followed by the above mentioned "tennis-ball" effect of newly formed CMP-NeuAc, our approach might be inadequate. However, such a cellular organization seems very illogical. Consequently, the possibility that the enzyme, CMP-NeuAc synthase, leaves the nucleus and exerts its action elsewhere in the cell becomes more attractive.

Coates *et al.* [8] have reported that enucleated mouse L 929 cells activate NeuAc to a lower extent than normal cells, and from these experiments they concluded that CMP-NeuAc synthase is functionally active in the nucleus. However, it is questionable whether CMP-NeuAc synthesis in such disordered cells can be compared to CMP-NeuAc synthesis *in vivo*. The possibility that CMP-NeuAc synthase is functionally active outside the nucleus has been suggested previously by Van den Eijnden [6] and Hughes [29]. Earlier Hultsch *et al.* [30] reported that a 105 000 × *g* supernatant of rat liver could easily synthesize CMP-NeuAc from exogenous NeuAc and CTP. Creek *et al.* [9] investigated the percentage activity present in the cytosolic and Golgi fraction. They found 1% of the enzyme activity localized in the Golgi fraction. One percent of the total CMP-NeuAc synthase is enough to provide the cell with a working level, because for whole rat liver the total enzyme capacity is about 200 nmol/min/g wet wt as measured *in vitro* [1] and the requirement is only 1 to 2 nmol/min/g wet wt [12].

If CMP-NeuAc synthase is functionally active in the cytosol, the interrelationship between this enzyme and the NeuAc-synthesizing enzymes might be comparable with that for other sugar-activating enzymes, with which no transport of NeuAc into, and CMP-NeuAc out of, the nucleus is required. The reason why this enzyme is stored in the nucleus and how its functioning is regulated remains a matter of speculation.

Acknowledgements

The electron microscopic analyses were done by Inge L. Eestermans, Dept. of Electron Microscopy (Chief: Prof. dr. E.C.M. Hoefsmit), Faculty of Medicine, Vrije Universiteit, Amsterdam. We thank Carin A.R.L. Govers and Piet L. Koppen for their technical assistance. The critical reading of the manuscript by Dr. A.P. Corfield (Dept. of Medicine, University of Bristol) is gratefully acknowledged.

References

1 van Rinsum J, van Dijk W, Hooghwinkel GJM, Ferwerda W (1983) Biochem J 210:21-28.
2 van Rinsum J, van Dijk W, Hooghwinkel GJM, Ferwerda W (1984) Biochem J 223:323-28.
3 Kean EL (1970) J Biol Chem 245:2301-8.
4 Gielen W, Schaper R, Pink H (1970) Hoppe Seylers Z Physiol Chem 351:768-70.
5 Gielen W, Schaper R, Pink H (1971) Hoppe Seylers Z Physiol Chem 352:1291-96.
6 van den Eijnden DH (1973) J Neurochem 21:949-58.
7 van Dijk W, Ferwerda W, van den Eijnden DH (1973) Biochim Biophys Acta 315:162-75.
8 Coates SW, Gurney T, Sommers LW, Yeh M, Hirschberg CB (1980) J Biol Chem 255:9225-29.
9 Creek KE, Weisman DC, Morre DJ (1980) Eur J Cell Biol 23:157-65.
10 Ferwerda W, Blok CM, Heijlman J (1981) J Neurochem 36:1492-99.
11 Ferwerda W, Blok CM, van Rinsum J (1983) Biochem J 216:87-92.
12 van Rinsum J, van Dijk W, Hooghwinkel GJM, Ferwerda W (1985) Glycoconjugate J 2:137-48.
13 Ferwerda W, Blok CM, van Rinsum J (1983) in Proc 7th Int Symp Glycoconjugates, eds. Chester MA, Heinegård D, Lundblad A, Svensson S, Secretariat, Lund p 733-34.
14 Siebert G (1961) Biochem Z 334:369-87.
15 Lisman JJW, de Haan J, Overdijk B (1979) Biochem J 178:79-87.
16 Blanken WM, Hooghwinkel GJM, van den Eijnden DH (1982) Eur J Biochem 127:547-52.
17 Balazs R, Cocks WA (1967) J Neurochem 14:1035-55.
18 Burton K (1956) Biochem J 62:315-23.
19 Munro HN, Fleck A (1966) in Methods Biochem Anal 16:145.
20 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265-75.
21 Warren L (1959) J Biol Chem 234:1971-75.
22 Ongun A, Stocking R (1965) Plant Physiol 40:825-34.
23 Elbers R, Heldt HW, Schmucker P, Soboll S, Wiese H (1974) Hoppe Seylers Z Physiol Chem 355:378-93.
24 Kimura H, Thomas E, Murad F (1974) Biochim Biophys Acta 343:519-28.
25 Sies H (1982) in Metabolic Compartmentation, ed. Sies H, Academic Press, London, p 1-6.
26 Reid E, El-Aaser ABA, Turner MK, Siebert G (1964) Hoppe Seylers Z Physiol Chem 339:135-49.
27 Richard M, Martin A, Louisot P (1975) Biochem Biophys Res Commun 64:108-14.
28 Berthillier G, Benedetto JP, Got R (1980) Biochim Biophys Acta 603:245-54.
29 Hughes RC (1976) in Membrane Glycoproteins, ed. Hughes RC, Butterworths, London, p 185.
30 Hultsch E, Reutter W, Decker K (1971) Biochim Biophys Acta 273:132-40.