Changes in the Hydroxyproline, Hexosamine and Sialic Acid of the Diabetic Human and β , β' -Iminodipropionitrile-Treated Rat Retinal Vascular Systems

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Summary. The retinal vasculature has been isolated from nondiabetic and diabetic post mortem human eyes by controlled trypsin digestion. Chemical analysis demonstrated increases in the hydroxyproline and hexosamine contents in diabetes. There was no general increase in the sialic acid content. These results have been related to histological preparations of sectors of the same retinas. Administration of β , β' -iminodipropionitrile to rats caused a retinopathy characterised by endothelial cell proliferation, increased PAS-positivity and microaneurysms. Chemical analysis of retinal vascular systems from β , β' -iminodipropionitrile-treated rats revealed increases in hydroxyproline, hexosamine and sialic acid contents.

Variations de l'hydroxyproline, de l'hexosamine et de l'acide sialique dans les systèmes vasculaires rétinien de l'homme diabétique et du rat traité par le β , β' -iminodipropionitrile

Résumé. Le système vasculaire rétinien a été isolé, par digestion trypsique contrôlée, après la mort, à partir d'yeux humains de non-diabétiques et de diabétiques. L'analyse chimique a révélé l'augmentation du contenu en hydroxyproline et en hexosamine, dans le diabète. Il n'y avait pas d'augmentation générale du contenu en acide sialique. Ces résultats ont été rapprochés des préparations histologiques de portions des mêmes rétines. L'administration de β , β' -iminodipropionitrile à des rats provoquait une rétinopathie caractérisée par une prolifération des cellules endothéliales, une PAS-positivité augmentée et des microanévrismes. L'analyse chimique des

systèmes vasculaires rétinien des rats traités par le β , β' -iminodipropionitrile, a révélé l'augmentation du contenu en hydroxyproline, en hexosamine et en acide sialique.

Änderungen des Gehaltes an Hydroxyprolin, Hexosamin und N-azetyl-Neuraminsäure in den Netzhautgefäßen von menschlichen Diabetikern und mit β , β' -Iminodipropionitril behandelten Ratten

Zusammenfassung. Die Netzhautgefäße wurden postmortal aus diabetischen und nichtdiabetischen Augen mit Hilfe einer kontrollierten Trypsin-Behandlung gewonnen. Bei der chemischen Analyse fand sich ein Anstieg des Hydroxyprolin- und Hexosamingehaltes in den diabetischen Augen. Der N-azetyl-Neuraminsäuregehalt war im allgemeinen nicht erhöht. Diese Resultate wurden in Beziehung gebracht zu den histologischen Befunden, die an den einzelnen Sektoren der gleichen Retina erhoben wurden. Verabreichung von β , β' -Iminodipropionitril an Ratten bewirkte eine Retinopathie unter den Zeichen einer Wucherung der Endothelzellen, verstärkter PAS-Anfärbbarkeit und Mikroaneurysmen. Die chemische Analyse der Netzhautgefäße von mit β , β' -Iminodipropionitril behandelten Ratten zeigte einen erhöhten Gehalt an Hydroxyprolin, Hexosamin und N-azetyl-Neuraminsäure.

Key-words: Human diabetic retinal vascular system, hydroxyproline, hexosamine, sialic acid, β , β' -iminodipropionitrile retinopathy, histology.

There have been numerous studies on the thickness of the basement membrane of the small blood vessels in diabetes mellitus. HARTMANN et al. (1959) showed an increase in the thickness of the diabetic glomerular membrane and this increase was related to the duration of the disease. These alterations in the structure and permeability of capillary basement membrane could be responsible for the sequelae of diabetic microangiopathy, such as exudates, endothelial proliferation, deposition of PAS-positive material and microaneurysms in the retina. However, not all workers have demonstrated changes in capillary basement membrane thickness. HANSEN (1965), FRIEDERICI et al. (1966) and McMILLAN et al. (1966) have reported no changes in the thickness of basement membrane from the peripheral glomerular basement membrane of juvenile diabetic kidney, the capillaries of the dorsal skin of the finger and the forearm skin capillaries of diabetics, respectively.

On the other hand, basement membrane thickening has been described in the capillaries of the pulp of the toe and finger by AGENAES and MOE (1961), of the glomerulus by BERGSTRAND and BUCHT (1957) and

DACHS et al. (1964) and of the forearm skin by HANDELSMAN et al. (1962). The general consensus of opinion is that basement membrane thickening does occur in diabetes but KIMMELSTIEL (1966) emphasized the importance of applying statistical methods to the interpretation of the observations and the dangers of including measurements of oblique sections of capillaries.

Capillary basement membrane disorder has been demonstrated electronmicroscopically in diabetic retinopathy by YAMASHITA and ROSEN (1962) and TOUSSAINT and DUSTIN (1963); YAMASHITA and BECKER (1961) have shown highly significant increases in the thickness of ciliary body basement membrane in diabetes. In order to elucidate further the chemical changes in the retina it was decided to determine the hydroxyproline, hexosamine and sialic acid content of normal and diabetic human retinal vascular systems after isolation by the method of KUWABARA and COGAN (1960).

Neuromotor disturbances in mice following the administration of β , β' -iminodipropionitrile (IDPN) were first reported by DELAY et al. (1952). SELYE (1957)

and FORGACS (1960) reported that this compound also caused severe ocular changes and HEATH and RUTTER (1966a) demonstrated that retinal capillary microaneurysms, endothelial proliferation and PAS-positive deposits developed in rats following a single dose of IDPN. These pathological changes in the rat retinal vascular system are similar to those which occur in human diabetes.

Estimations were therefore also carried out on retinal vascular systems isolated from normal and IDPN-treated rats.

Methods

Human eyes were obtained within 3 hours of death and placed in either sterile liquid paraffin and kept at 4°C until no longer required for corneal grafts and then transferred to 10% formol saline, or placed directly in the latter medium.

Male albino rats, 150–200 g maintained on M. R. C. diet 41 and water ad lib., were injected subcutaneously with a 40% aqueous solution of IDPN at a dose level of 200 mg/100 g body weight. The animals were maintained for 14 days on the normal diet, but with cortisol dissolved in the drinking water at such a concentration that each rat took approximately 1 mg cortisol/day. They were killed by cervical dislocation and the eyes were enucleated and placed in 10% formol saline.

digested at 65°C for 18 hours in 0.5 ml of a medium consisting of 0.1 M phosphate buffer, pH 6.5, containing 5 mM EDTA, 5 mM cysteine hydrochloride, 0.1 M NaCl and 50 µg crystalline papain. Weighed aliquots of the clear digest (0.1 ml, 0.2 ml and 0.2 ml, respectively) were taken for the estimation of hydroxyproline content by the method of STEGEMANN (1958) after 6 hours hydrolysis in 6 N HCl at 100°C, of hexosamine content by the modified Elson and Morgan reaction adopted by ANTONOPOULOS et al. (1964) after 8 hours hydrolysis in 6 N HCl at 100°C, and of sialic acid content by the thiobarbituric acid method of WARREN (1959) after the aliquot, dried over P₂O₅, had been hydrolysed in 0.2 ml 0.1 N H₂SO₄ at 80°C for 1 hour. The HCl used in the first two estimations was removed after hydrolysis in a vacuum desiccator over NaOH flake.

Results

The hydroxyproline content of the human retinal vascular system was significantly ($p < 0.0005$) increased in the diabetic state from a mean nondiabetic value of 26.0 (Table 1) to a mean value of 53.9 mg/g dry weight. This increase was to a certain extent related to the severity of the retinopathy. Case O.D. was a man aged 60 years who died from respiratory insufficiency and case J.B. was a man aged 76 years with late

Table 1. *Hydroxyproline, hexosamine and sialic acid contents of human retinal vascular system*

	Age	Hydroxyproline mg/g dry weight	Hexosamine mg/g dry weight	Sialic acid mg/g dry weight
Nondiabetic				
Mean (21) ± SEM		26.00 ± 1.65	18.00 ± 1.67	11.30 ± 1.39
Diabetic O.D. R	60	51.90	30.90	11.10
L		45.40	35.00	10.30
Diabetic J.B. R	76	49.40	26.00	13.20
L		52.10	24.50	12.60
Diabetic S.W. R	56	50.40	23.10	15.00
L		54.10	27.30	17.60
Diabetic W.H.	37	73.80	39.40	
Mean (7) ± SEM		53.90 ± 3.48	29.50 ± 2.26	13.30 ± 2.67

The figures in parenthesis represent the number of retinas.

After the eyes had been stored for at least 48 hours in formol saline, retinas were dissected out. The vascular system was isolated from a small sector of each human retina and from individual rat retinas using the technique of differential digestion with trypsin (KUWABARA and COGAN, 1960) and stained with periodic acid-Schiff (PAS) and haematoxylin.

The vascular systems were isolated in a similar fashion from the remainder of each human retina and 30 rat retinas. The weights of each individual human retinal vascular and the combined 30 rat retinal vascular systems were determined on an Oertling microanalytical balance by drying to a constant weight over P₂O₅ in a vacuum desiccator. These were then

maturity onset diabetes who died from pneumonia. Histological examination of the retinal vascular systems from these two cases showed the presence of a normal structure with some peripheral avascularity, but capillary microaneurysms and PAS-positive deposits were absent. The hydroxyproline contents were, however, considerably increased above the nondiabetic value. Case S.W. was a man aged 56 years with a duration of diabetes of 13 years, who died of uraemia. Histopathological examination revealed a moderately severe retinopathy with some areas of endothelial cell proliferation and associated PAS-positive deposits and a few microaneurysms. The hydroxyproline content of this vascular system was

approximately double that of the mean nondiabetic value and was of the same order of magnitude as found in cases J.B. and O.D. Case W.H. was a woman aged 37 years with a duration of diabetes of 31 years, who died of renal failure. Both eyes showed signs of retinitis proliferans and it was not possible to isolate the intact retinal vascular system from each eye. Those portions of the retinal vascular system which did not contain newly formed blood vessels were combined for analysis. The hydroxyproline content was 73.8 mg/g dry weight compared with the mean value of 53.9 for the diabetics and 26.0 for the nondiabetics. The highest hydroxyproline content of the vascular systems from the nondiabetic cases was found to be 38.3 mg/g dry weight. A few peripheral microaneurysms were seen on histological examination. This eye was obtained from an eye bank and a case history was not available, other than a cause of death of bronchopneumonia.

The hydroxyproline content of the 30 pooled normal rat retinal vascular systems was 22.8 mg/g dry weight (Table 2), which is of the same order of magnitude as the value for the nondiabetic human systems. Fourteen days after the administration of IDPN the hydroxyproline content had increased to 31.8 mg/g dry weight. The retinas of these rats exhibited a retinopathy characterized by endothelial proliferation, PAS-positive deposits and the presence of scattered microaneurysms. Neovascularization was not present.

Table 2. *The hydroxyproline, hexosamine and sialic acid contents of normal and IDPN-treated rat retinal vascular system*

Animals	No. of eyes	Hydroxyproline mg/g dry weight	Hexosamine mg/g dry weight	Sialic acid mg/g dry weight
Normal	30	22.8	32.0	12.2
IDPN-treated ¹	30	31.8	85.3	39.2

¹ 14 days after treatment with 200 mg IDPN/100 g body weight.

The hexosamine content of all the diabetic retinal vascular systems was significantly greater than the mean normal value of 18.0 mg/g dry weight, compared with a mean diabetic value of 29.5 ($p < 0.0005$). There was, however, no statistical significance between the mean values of 11.3 and 13.3 mg sialic acid/g dry weight for the normal and diabetic human retinal vascular systems.

There were 170% and 220% increases in the hexosamine and sialic acid contents of the IDPN-treated rat retinal vascular systems, from normal values of 32.0 to 85.3 and 12.2 to 39.2 mg/g dry weight respectively.

Discussion

Glomerular basement membrane has been isolated from normal and diabetic human kidneys by differential

centrifugation to separate glomeruli, followed by treatment with 0.05 N NaOH for the removal of cell debris (LAZAROW and SPEIDEL, 1964). The isolated membrane was shown to be strongly PAS-positive and chemical analysis revealed that it consisted of a hydroxyproline-rich, collagen-type protein associated with 3.4% carbohydrate. Equal quantities of glucose and galactose were the major components of this carbohydrate with small amounts of mannose and fucose; only traces of hexosamine were present. The chemical composition of basement membrane isolated from diabetic kidneys did not differ from that of normal tissue, although the hydroxyproline content of the whole glomeruli was considerably increased.

Although it is possible to isolate sufficient quantities of basement membrane from kidney glomeruli for chemical analysis, the isolation of even small blood vessels from other tissues affected by diabetic microangiopathy is difficult to achieve in sufficient quantity and purity for chemical determination. KUWABARA and COGAN (1960) have shown that the neural tissue of the retina can be selectively digested by controlled treatment with trypsin, making it possible to isolate the retinal vascular system for histological examination. Advantage has been taken of this method for the isolation of the retinal microvasculature and chemical analyses have revealed a significant increase in the hydroxyproline content of the diabetic retinal vascular system.

YAMASHITA and BECKER (1961) have demonstrated that ciliary body basement membrane increases in thickness in normal subjects with age, from approximately 1 μ to 4 μ , but the mean value in diabetics with retinopathy was found to be 10.1 μ . Case W.H. was 37 years old and younger than the other diabetic subjects in this study and the high content of hydroxyproline must therefore have been due to the pathological condition of the retina.

SPEIDEL and LAZAROW (1964) have found that glomerular basement membrane contains 12% hydroxyproline and based on this figure the most severely diabetic retinal vascular system contained approximately 60% of the dry weight as basement membrane, whereas the nondiabetic retinal vasculature contained approximately 20%.

There was a general increase in the hexosamine content of the diabetic retinal vascular systems but glomerular basement membrane contains only traces of hexosamine, thus these observed changes would appear to be associated with a constituent of the retinal vascular system other than the basement membrane. No significant changes were found in the sialic acid content of the diabetic retinal vascular systems.

Many investigators have studied the effects of the lathyrogen β -aminopropionitrile (BAPN) on collagen metabolism (c.f. TANZER, 1965). Treatment of experimental animals with this substance causes an increase in the amount of extractable collagen due to a weakening of the inter- and intramolecular cross-linkages,

resulting in tissue fragility and death in young rats due to dissecting aortic aneurysm. In an attempt to accelerate the formation of retinopathy in alloxan-diabetic rats, RUTTER and HEATH (1965) administered BAPN to these animals; no changes were, however, observable in the retina. IDPN, a compound related to BAPN, caused a severe retinopathy, characterized by endothelial cell proliferation, PAS-positive deposits and microaneurysms (HEATH and RUTTER, 1966a, b). Chemical analysis of 30 pooled retinal vascular systems from rats, treated 14 days previously with a single dose of IDPN, showed a 40% increase in the content of hydroxyproline. This amino acid occurs only in collagenous-type proteins and the increase would not have been observed if the PAS-positive deposits consisted solely of aggregates of plasma glycoprotein. The latter, however, were certainly also present since considerable increases occurred in the hexosamine and sialic acid contents of retinal vascular systems from IDPN-treated rats. Histological examination of these vascular systems showed a generalised increase in the PAS-positivity of the vessel walls, together with the presence of scattered amorphous PAS-positive deposits. Inducement of a retinopathy in experimental animals by the administration of IDPN may provide a means for understanding the mechanism of endothelial cell proliferation and microaneurysm formation. The biochemical action of this compound is being further investigated.

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References

- AAGENAES, Ø., and H. MOE: Light- and electronmicroscopic study of skin capillaries of diabetics. *Diabetes* **10**, 253–259 (1961).
- ANTONOPOULOS, C.A., S. GARDELL, J.A. SZIRMAI and E.R. DE TYSSONSK: Determination of glycosaminoglycans (mucopolysaccharides) from tissues on the microgram scale. *Biochim. biophys. Acta* **83**, 1–19 (1964).
- BERGSTRAND, A., and H. BUCHT: Electron microscopic investigations on the glomerular lesions in diabetes mellitus (diabetic glomerulosclerosis). *Lab. Invest.* **6**, 293–300 (1957).
- DACHS, S., J. CHURG, W. MAUTNER and E. GRISHMAN: Diabetic nephropathy. *Amer. J. Path.* **44**, 155–168 (1964).
- DELAY, J., P. PICHOT, J. THUILLIER and J-P. MARQUSET: Action de l'amino-dipropionitrile sur le comportement moteur de la souris blanche. *C. R. Soc. Biol.* **146**, 533–534 (1952).
- FORGACS, J.: Les altérations oculaires dans le lathyrisme expérimental dues à l'imino-dipropionitrile (IDPN). *Arch. ophthalm.* **20**, 275–284 (1960).
- FRIEDERICI, H.H.R., W.R. TUCKER and T.B. SCHWARTZ: Observations on small blood vessels of skin in the normal and in diabetic patients. *Diabetes* **15**, 233–250 (1966).
- HANDELSMAN, M.B., T.G. MORRIONE and B. CHITMAN: Skin vascular alterations in diabetes mellitus. *Arch. intern. Med.* **110**, 70–77 (1962).
- HANSEN, R.O.: A quantitative estimate of the peripheral glomerular basement membrane in recent juvenile diabetes. *Diabetologia* **1**, 97–100 (1965).
- HARTMANN, J.F., F.C. GOETZ and A. LAZAROW: Electron microscopy of the human kidney in diabetes. In OBERDISSE, K. and K. JAHNKE (editors): *Proc. III int. Diabetes Fed.* Stuttgart, Georg Thieme, p. 189–191, 1959.
- HEATH, H., and A.C. RUTTER: Retinal angiopathy in the imino-dipropionitrile-treated alloxan-diabetic rat. *Brit. J. exp. Path.* **47**, 116–120 (1966a).
- Imino-dipropionitrile retinopathy in the rat. In GRAHAM-JONES, O. (editor): *Aspects of Comparative Ophthalmology*. Oxford, Pergamon Press, p. 317–328, 1966b.
- KIMMELSTIEL, P.: Basement membrane in diabetic glomerulosclerosis. *Diabetes* **15**, 61–63 (1966).
- KUWABARA, T., and D.G. COGAN: Studies of retinal vascular patterns. Part 1. Normal architecture. *Arch. ophthalm.* **64**, 904–911 (1960).
- LAZAROW, A., and E. SPEIDEL: The chemical composition of the glomerular basement membrane and its relationship to the production of diabetic complications. In SIPERSTEIN, M.D., A.R. COLWELL, Sr., and K. MAYER (editors): *Small Blood Vessel Involvement in Diabetes Mellitus*. Washington, D.C., American Institute of Biological Sciences, p. 127–150, 1964.
- McMILLAN, D.E., D.L. BREITHAUP, W. ROSENAU, J.C. LEE and P.H. FORSHAM: Forearm skin capillaries of diabetic, potential diabetic and nondiabetic subjects. Changes seen by light microscope. *Diabetes* **15**, 251–257 (1966).
- RUTTER, A.C., and H. HEATH: The retinal vascular system in experimental diabetes. In GRAYMORE, C.N. (editor): *Biochemistry of the Retina*. London, Academic Press, p. 139–142, 1965.
- SELYE, H.: Lathyrism. *Rev. canad. Biol.* **16**, 1–82 (1957).
- STEGEMANN, H.: Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-Dimethylaminobenzaldehyd. *Hoppe-Seylers Z. physiol. Chem.* **311**, 41–45 (1958).
- TANZER, M.L.: Experimental lathyrism. In HALL, D.A. (editor): *International Review of Connective Tissue Research*, vol. 3. New York, Academic Press, p. 91–112, 1965.
- TOUSSAINT, D., and P. DUSTIN: Electron microscopy of normal and diabetic retinal capillaries. *Arch. ophthalm.* **70**, 96–108 (1963).
- WARREN, L.: The thiobarbituric acid assay of sialic acids. *J. biol. Chem.* **234**, 1971–1975 (1959).
- YAMASHITA, T., and B. BECKER: The basement membrane in the human diabetic eye. *Diabetes* **10**, 167–174 (1961).
- , and D.A. ROSEN: Electronmicroscopic study of diabetic capillary aneurysm. *Arch. ophthalm.* **67**, 785–790 (1962).

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