Table 2. Percent increase in reducing sugar, starch and protein content in green islands[a] formed on mustard leaf as compared to the surrounding tissue

Nutrient	Green island	Surrounding tissue	Percent increase as compared to surrounding yellowed tissue
A. in green islands formed under infection-drops			
Reducing sugar (O.D. at 665 nm)	0.68	0.16	325.0
Starch (O.D. at 665 nm)	0.25	0.10	150.0
Protein (O.D. at 660 nm)	0.17	0.11	50.0
B. in green islands formed under drops of cytokinin extracted from culture filtrate			
Reducing sugar (O.D. at 665 nm)	1.50	0.16	837.5
Starch (O.D. at 665 nm)	0.34	0.10	240.0
Protein (O.D. at 660 nm)	0.19	0.10	90.0

[a] After 72 h incubation in dark at 30 °C.

tein were estimated by the methods of Yem and Willis[9] and Lowry et al.[10] respectively.

Results and discussions. Taking the original concentration of extract to be 100%, bioassays were performed with 100, 50 and 25% concentrations of extract obtained from green islands/yellowed tissue/culture filtrate. The results indicated that there was 79.54, 59.84, 43.18% inhibition in root length of barley seedlings over blank; 120.61, 53.49, 27.51% increase in fresh weight of cotyledons over blank; 128.69, 65.02, 33.18% increase in area of radish cotyledons over blank; and 650, 270, 130% chlorophyll retention in cotyledons over blank when different dilutions of cytokinins, extracted from green islands formed on leaf tissue under infection drops containing germinating conidia of *A. brassicicola*, were bioassayed. When results of the above tests were compared with the calibration curve the concentration of the active substance in the green island extract was found to be equivalent to 47 µg/ml of kinetin. Except for a little inhibition in root length of barley seedlings, the results of other bioassays were the same as in the blank when bioassays were performed with cytokinins extracted from yellowed tissue.

When cytokinins extracted from culture filtrate of *A. brassicicola* were bioassayed, 90.67, 36.54, 16.04% inhibition in root length of barley seedlings was obtained over blank; 289.42, 27.81, 10.37% increase was obtained in fresh weight of radish cotyledons over blank; 289.82, 27.87, 10.61% increase in area of radish cotyledons was obtained over blank; and 17500, 110, 30% chlorophyll retention in cotyledons over blank was observed. When results of the above tests were compared with the calibration curve, concentration of the active substance in the extract was found to be equivalent to approximately 119 µg/ml of kinetin.

All bioassays show the presence of cytokinins in the green islands formed on detached mustard leaves at areas beneath the infection-drops containing germinating conidia of *A. brassicicola*. Since the leaves had been detached, it is obvious that cytokinins in the green islands must have been secreted by the pathogen. This is confirmed by the secretion of cytokinins by the fungus in liquid synthetic medium. Moreover, treatment of mustard leaf (healthy, detached) with cytokinins extracted from the culture filtrate of *A. brassicicola* evoked the formation of green islands after 72 h incubation in dark.

Since cytokinins delay senescence in excised leaves[11-13], it appears that the cytokinins secreted by the germinating conidia in the infection-drops and in the culture medium lead to the retardation of senescence in the leaf tissue. It is clear from all that has been discussed above that cytokinins secreted by the germinating conidia of *A. brassicicola*, like those from *Helminthosporium turcicum*, may create localized translocatory sinks at quite an early stage. The nutrients move towards these sinks from the surrounding areas and supply food and energy to the growing pathogen for penetration and for further development after penetration.

This was confirmed by actual quantitative estimation of sugar, starch and protein content in green islands. The sugar, starch and protein contents were 325, 150, 50% more in green islands formed beneath the infection-drops of *A. brassicicola* than in the surrounding tissue. In green islands formed under the drops of cytokinin extracted from culture filtrate of *A. brassicicola* sugar, starch and protein contents were 837.5, 240, and 90% respectively more than in the surrounding tissue (table 2).

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The elimination of 1,5-anhydroglucitol administered to rats

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Summary. Rat serum contains natural 1,5-anhydroglucitol. Injected or orally administered 1,5-anhydroglucitol was efficiently reabsorbed by the renal tubuli via a mechanism which had a saturation point at high serum 1,5-anhydroglucitol levels. The compound had a slow turnover rate in the body; its half-life is approximately 3 days. The compound was readily absorbed in the gut when administered orally.

1,5-anhydroglucitol (AG) is one of the major polyols of human cerebrospinal fluid[1,2] and plasma[3-5]. AG is a nonreducible C_6-monosaccharide which appears to be freely filterable through the renal glomeruli. Studies in humans have suggested that the renal tubular cells reabsorb AG, since no AG has been found in the urine[5]. An association has been discovered between

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changes in the AG level in both cerebrospinal fluid and serum, and certain pathological conditions. Very low levels of AG occur in human diabetes mellitus[1,3-5]. Only meagre data are available on the origin and fate of AG in the organism. The role of AG in body metabolism is completely unknown. The aim of this study was to obtain some fundamental data on the fate of AG administered to rats.

Materials and methods. White laboratory rats weighing 200–330 g were used. In short-term experiments 2–7 mg AG was given orally, i.p. or i.m. in a small volume of saline. In a further experiment 7 mg AG (0.14 mmol/kg body weight) was given daily to each of 12 rats orally in drinking water for 7 weeks. 6 rats from the same batch served as controls.

Tail tip blood was collected in heparinized glass capillary tubes. The blood samples were transferred into small centrifuge tubes and plasma was collected after centrifugation. Metabolic cages were used for the collection of urine. The AG level in plasma and urine was determined by means of gas-liquid chromatography[5] with capillary glc-columns of Silar 10 C (Oriola Co, Espoo, Finland). Ribitol was the internal standard. The mass spectrum analysis of the compound was carried out as described earlier[5]. A hexokinase method (Gluco-quant, Boehringer, Mannheim, Germany) was used for determination of whole blood glucose level.

Results and discussion. The AG level in the serum of untreated rats was 47 ± 24 (SD) µmol/l (N = 11). The identity of the compound was ascertained by the specific retention time in glc and by the typical fragmentogram obtained in mass spectrum analysis[1]. No AG was found in urine. The data suggest that AG is efficiently reabsorbed by rat kidney tubuli, as is also the case in humans[5].

After i.p. or i.m. administration the AG level in serum rapidly increased and a part of the AG given was recovered in urine (fig. 1, table 1). This indicates that the reabsorption capacity is saturable. The threshold level of AG in serum, above which AG was excreted, was 95 ± 36 µmol/l (N = 7). Of 5 mg AG administered (15.1–16.7 mg/kg body weight) 0.6–1.4 mg was recovered in the urine during the first 24 h, after which urinary excretion of AG was negligible (table 1).

Table 1. Recovery in urine of 1,5-anhydroglucitol given to rats

Amount given		Rat	mg excreted Day 1	Day 2	Day 3
i.p.	5 mg	1	1.2	0	0
	5 mg	2	0.6	0	0
	5 mg	3	1.4	0	0
i.m.	2.5 mg	4	0.2	0	0
p.o.	2 mg	5	0.3	0	0
	5 mg	6	1.1	0.3	0
	5 mg	7	1.2	0.3	0
	5 mg	8	1.2	0.4	0
	7 mg	9	3.1	0.3	0

However, after 24 h of administration the AG level in serum still exceeded the pre-administration level. The level dropped slowly and the preadministration level was reached in 4–7 days. T½ of the AG administered was 1.6–5.2 days (mean 74 h, N = 5).

The serum AG level also increased rapidly after oral administration. The peak concentration in serum was noted 1 h after the administration (fig. 2). This suggests that the compound was readily absorbed by the gut. Of 5 mg AG given, 1.4–1.6 mg was recovered in urine in 48 h after which there was no urinary excretion of AG (table 1). T½, measured after the urinary AG excretion ceased, was 2.4–3.1 days (mean = 64 h, N = 4). The renal threshold value was 104 ± 34 µmol/l (N = 4). A high serum AG level (62–126 µmol/l) was maintained in the 12 rats to which AG was given orally for 7 weeks, compared with the level of 24–62 µmol/l in the 6 control rats

Table 2. Serum 1,5-anhydroglucitol (AG) in the rat

Normal level	47 ± 24 (SD) µmol/l (N = 11)
T½ of AG administered	2.9 ± 1.2 days (N = 9)
Renal threshold level	98 ± 36 µmol/l (N = 11)
Peak serum concentration	1 h after oral administration of AG
Peak urinary excretion	During the first 24 h after AG administration

to which no AG was given. The AG administration produced no apparent toxic symptoms in the test animals. Their weight gain (5.2 g/week/rat) was similar to the weight gain of the control rats (4.6 g/week/rat). The fasting blood glucose level was also similar in both groups at the end of the test period (3.8–6 mmol/l, compared to 3.8–6.1 mmol/l in the control group).

The present data tally with the results of an earlier study[6] in which oral administration of AG was not associated with any changes in the fasting respiratory quotient, or in the storage of glycogen (in rats) or in changes in the fasting blood sugar level (in rabbits). On the other hand, acute intraventricular infusion

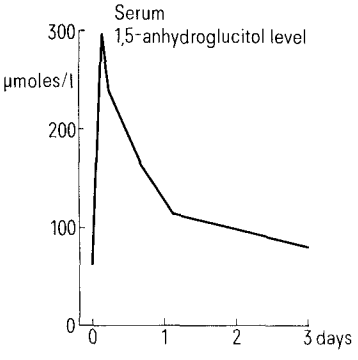

Figure 1. Serum 1,5-anhydroglucitol (AG) concentration following intramuscular administration of 5 mg (92 µmol/kg) AG at time 0 in a rat.

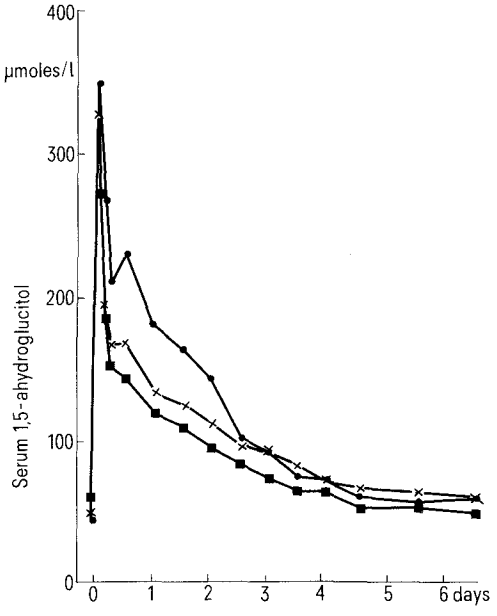

Figure 2. Serum 1,5-anhydroglucitol (AG) concentration following oral administration of 5 mg (139–153 µmol/kg) AG at time 0 in 3 rats.

of AG (rat brain) has been reported[7] to produce hyperglycemia. The rather long T½ time noted in the present study (table 2) indicates that the turnover rate of AG in the body is slow, and suggests that the compound is not an energy metabolite.

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Binding of centchroman – a nonsteroidal antifertility agent to human plasma proteins[1]

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Summary. Centchroman, a non-steroidal antifertility agent showed a low affinity ($K_d = 13.19 \times 10^{-6}$ M) and nonsaturable binding to human plasma. Centchroman did not compete either with sex hormone binding globulin or corticosteroid binding globulin. Polyacrylamide gel electrophoresis and temperature dependent binding characteristics revealed that the protein responsible for centchroman binding to human plasma resembles albumin.

Contraceptive efficacy of centchroman [3,4-trans-2,2-dimethyl-3-phenyl-4 (p-pyrrolidinoethoxy-phenyl)-7-methoxy chroman hydrochloride], a non-steroidal antifertility agent, has been evaluated by Kamboj and his associates[2-3]. Centchroman binds to the estrogen receptor in rat uterus, which has been suggested as its molecular site of interaction[4]. However, the nature of centchroman binding to steroid binding proteins in plasma is yet unknown. The binding of contraceptive steroids to plasma proteins such as sex hormone binding globulin (SHBG), cortisol binding globulin (CBG) and albumin has been reported recently[5,6]. It is widely accepted that the unbound fraction of steroid in blood is biologically available to the target cells for its interaction with the receptor sites[7]. Therefore, in the present study, the binding of centchroman to steroid binding proteins in human plasma has been investigated using charcoal adsorption and polyacrylamide gel electrophoresis.

Methods. ^{14}C-centchroman (sp. act. 2.932 mCi/mmol) and unlabelled centchroman synthesized at this Institute were used. ^{3}H-Estradiol-17β, (sp. act.-110 Ci/mmol) and ^{3}H-Cortisol (sp. act.-114.5 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts, USA. Authentic steroid standards obtained by Prof. D.N. Kirk, Steroid Reference collection (Great Britain) were used. Purified human serum albumin (HSA) was procured from Sigma Chemical Co., USA.

Blood was drawn from healthy women and plasma was obtained by centrifugation at 800 × g for 15 min. To remove endogenous steroids, the plasma was treated with dextran-coated charcoal (DCC) suspension (0.5 g activated charcoal and 0.05 g of dextran/100 ml of Tris-HCl buffer, pH 7.4) for 15 min at 4 °C. Prior to the binding assay plasma was diluted (1:1) with 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 1 mM mercaptoethanol and 10% glycerol. 100 µl of diluted plasma (in duplicate) was incubated with ^{14}C-centchroman (2 µM to 60 µM) for 2 h at 4 °C in the absence or presence of a 1000-fold molar excess of unlabelled centchroman. Following incubation, protein-bound and free fractions were separated by addition of 500 µl of DCC suspension. After centrifugation at 800 × g for 10 min at 4 °C, the radioactivity was determined in the supernatant (bound fraction) as described earlier[7].

For competitive binding, incubations were carried out in the presence of a 1000-fold molar excess of unlabelled competitors using ^{14}C-centchroman, ^{3}H-estradiol and ^{3}H-cortisol as radioligands.

To study the effect of protein concentration on centchroman binding, the assay was carried out with diluted plasma in the ratio of 1:1, 1:5, 1:10, 1:20 and 1:50. The binding assay was carried out as described above. The effect of temperature on binding was studied by incubating centchroman with plasma at different temperatures of 4, 45 and 60 °C.

The characterization of centchroman bound complex was accomplished by polyacrylamide gel electrophoresis (PAGE). 100 µl of human serum albumin in buffer (50 mg/ml) or 100 µl of human plasma was incubated with ^{14}C-centchroman in the absence and presence of a 1000-fold molar excess of unlabelled centchroman at 4 °C and then subjected to PAGE at 4 °C as described earlier[9]. Following the run, gels were cut into 2 mm thick slices and radioactivity was assessed.

Results and discussion. Studies with ^{14}C-centchroman showed a non-saturable and low affinity binding in human plasma. The Scatchard analysis of binding data revealed a dissociation constant (K_d value) in the range of 13.19×10^{-6} M indicating low affinity and high capacity binding to human plasma (fig. 1). Incubations in the presence of unlabelled centchroman (1000-fold molar excess) failed to saturate the binding sites.

The results on binding studies in the various dilutions of human plasma revealed that ^{14}C-centchroman binding increased

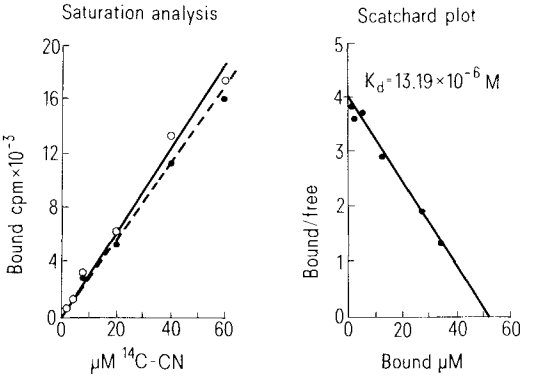

Figure 1. Saturation and Scatchard Plot analysis of centchroman (CN) binding in human plasma. 100 µl of diluted (1:1) plasma was incubated with increasing mass of ^{14}C-CN in the absence (○——○) or presence (●——●) of unlabelled CN at 4 °C for 2 h. Following incubation, bound and free CN were separated by DCC (see text).