Formation of radioactive gallic acid in *Rhus* and *Acer* leaves from labelled compounds

Compounds fed	Metabolic period (h)	Leaf age	Radioactivity (dpm) of 0.6 ml of the MeOH-HCl leaf-extract of		Specific activity (dpm/μMol) of gallic acid		Distribution[a] of radioactivity in gallic acid (%)	
			Rhus	*Acer*	*Rhus*	*Acer*	*Rhus*	*Acer*
Phenylalanine-U-^{14}C	7	Juvenile	21865	—	414	—	0.16	—
	23	Juvenile	51599	—	417	—	0.23	—
	7	Mature	10914	16848	217	417	0.24	0.31
	23	Mature	10789	18977	322	705	0.42	0.46
Shikimic acid-U-^{14}C	7	Juvenile	91475	—	206	—	0.04	—
	23	Juvenile	93666	—	556	—	0.11	—
	7	Mature	26468	25787	898	380	0.53	0.22
	23	Mature	16513	22213	2649	853	2.46	0.51

[a] The radioactivity (dpm) of 0.6 ml of the MeOH-HCl extract (total vol., 10 ml) was regarded as 100%.

a definite period, each sample was rinsed in water, blotted and weighed. The cut-up leaves were dropped into boiling 0.5% methanol-HCl, heated for a few minutes and then cooled with tap water. The extract was decanted and the residue thoroughly extracted with the same solvent until it became colourless. The combined extract was accurately made up to 10 ml and 0.6 ml aliquots were concentrated to a small volume, spotted on paper and a two-dimensional chromatogram prepared by developing first in *n*-BuOH – HOAc – H_2O (6:1:2) and then 6% acetic acid. The chromatograms were air-dried and examined in UV-light (256 nm) before and after exposure to ammonia vapour when gallic acid and other compounds were detected. The identification of gallic acid has been mentioned in a previous report[5]. Then the chromatograms were covered X-ray film (Sakura, non-screen type) and exposed for 36 days. The autoradiograms obtained were compared with the spots observed above, and the spot containing gallic acid was cut from the chromatogram and put in a vial containing 10 ml of scintillator, which consisted of the mixture of toluene — 2, 5-diphenyloxazole (DPO) – 1, 4-*bis*(2-methylstyryl)-benzene — triton X-100 (3 l:10 g: 1.5 g: 1.5 l). The radioactivity of gallic acid was measured in a Packard liquidscintillation spectrometer Model 3385. A blank area of the chromatogram, equivalent to the gallic acid spot, was used as control. The gallic acid in the eluate from the chromatograms was measured spectrophotometrically at 274 nm and quantitatively determined by comparison with the standard curve of the authentic specimen.

When phenylalanine-^{14}C or shikimic acid-^{14}C was infiltrated into leaves, the incorporation rate of ^{14}C of both precursors into gallic acid was found to be characteristic of the species *Rhus* or *Acer* and the age of their leaves. As shown in the Table, the mature leaves of *Acer buergeranum* utilized phenylalanine and shikimic acid as good precursors for producing gallic acid at the about same rate. Unfortunately, gallic acid in the juvenile *Acer* leaves was insufficiently recovered for the tracer analysis. On the other hand, in the juvenile leaves of *Rhus succedanea*, the incorporation rate (as %) of phenylalanine-^{14}C into gallic acid was about 2 or 4 times that of shikimic acid-^{14}C. In contrast, phenylalanine was effective by only about $^1/_2$ to $^1/_6$ as much as shikimic acid for the synthesis of gallic acid in the mature *Rhus* leaves. Thus the formation of gallic acid in the *Acer* leaves seems to proceed both from direct dehydrogenation of shikimic acid and β-oxidation of the phenylpropanoid at the mature stage. On the other hand, the biosynthesis of gallic acid in the *Rhus* leaves might proceed preferentially by β-oxidation of the phenylpropanoid derived from phenylalanine at the juvenile stage, and in preference by dehydrogenation of shikimic acid at the mature stage, respectively.

Summary. The incorporation of ^{14}C-labelled phenylalanine and shikimic acid into gallic acid by the leaves of *Rhus succedanea* and *Acer buergeranum* suggested that two alternative pathways could exist for the biosynthesis of gallic acid, and that the preferential route for the acid formation seems to be influenced by leaf age and species of the plants.

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Increased Collagen and Glycoprotein Contents of the Denervated Cremaster Muscle of the Bonnet Monkey, *Macaca radiata*

Transection of genitofemoral nerve results in an increase in alkali-soluble collagen and in a progressive atrophy of the monkey cremaster muscle[1]. However, the pool size of neutral salt- and acid-soluble and insoluble[2] collagens of the denervated muscle are not known. We have examined whether this increase in collagen, due to denervation atrophy, was reflected uniformly in all types of collagens.

The animal care and surgical procedure for transection of the genitofemoral nerve in adult male bonnet macaques,

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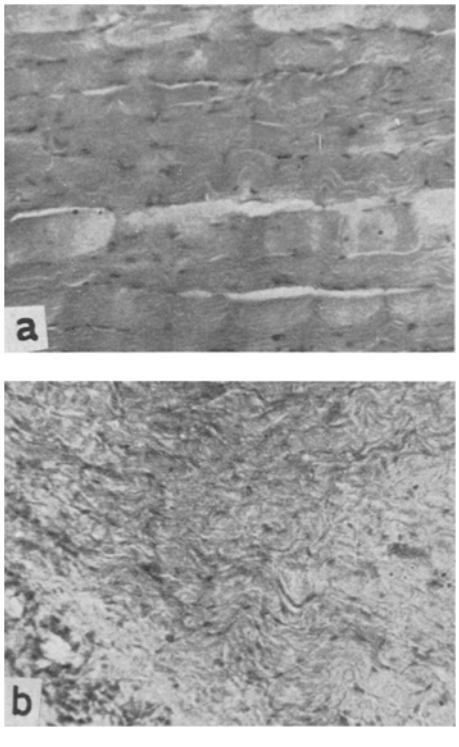
Macaca radiata, was similar to that described previously[1]. 60 days after nerve transection, the cremaster muscle was isolated from the anaesthetised animals and divided into 2 unequal transverse halves; the smaller one was used for histological examination, while the other was weighed and used for collagen extraction. The muscles from normal animals served as controls. Collagenous fraction of the muscle was isolated after extracting sarcoplasm and contractile proteins[3]. The neutral salt-, acid- and alkali-soluble and insoluble collagens were fractionated according to the rationale described by JACKSON and CLEARY[4]. The protein of each fraction was estimated by microkjeldahl method. The hydroxyproline content, on hydrolyzing the fraction in 6 *N* HCl for 3 h at 12 lbs pressure in an autoclave, was estimated by Woessner's method[4]. The glycoproteins were extracted and estimated according to the method of Weimer and Moshin[5].

Table I. Distribution of neutral salt-, acid- and alkali-soluble and insoluble collagens in the denervated cremaster muscle of bonnet monkey

Fraction	mg collagen/g wet muscle		Incidence of change on denervation
	Normal	Denervated	
Neutral salt-soluble	3.68 ± 0.24	9.19 ± 0.31	Increase $p < 0.001$
Acid-soluble	0.98 ± 0.08	0.88 ± 0.06	No change $p > 0.01$
Alkali-soluble	68.71 ± 1.78	92.68 ± 2.01	Increase $p < 0.001$
Insoluble collagens	25.14 ± 1.31	54.46 ± 1.08	Increase $p < 0.001$
Glycoproteins (mg hexose/g protein)	3.13 ± 0.98	8.62 ± 1.03	Increase $p < 0.001$

Values are $\bar{X}$ ± SD of 6 observations.



Low power (×70) photomicrograph of normal (a) and denervated (b) cremaster muscle of bonnet monkey; sectioned longitudinally and stained in iron-hematoxylin.

The longitudinal paraffin sections of the denervated muscle stained with iron-hematoxylin showed accumulation of connective tissue (Figure), which mainly constitutes the collagens and glycoproteins in the muscle. Fractionation of the collagenous materials revealed (Table I) that denervation increased the alkali- and neutral salt-soluble and insoluble collagens, whereas the acid-soluble collagen content remained unchanged on denervation. The glycoproteins significantly increased on denervation (Table I). The hydroxyproline content did not vary in the acid- and alkali-soluble collagens, but increased significantly in neutral salt-soluble and insoluble collagens (Table II).

These results demonstrate that the denervation of the cremaster muscle impairs the total turnover of different collagen fractions; since neutral salt-soluble collagen are known to be the precursors[6,7] for insoluble collagens. The solubility of collagens in neutral salt solutions, acids and alkalis varies as the molecules undergo intramolecular and intermolecular cross-links and simultaneously mature to attain a state of insolubility and metabolic inertness[2]. The variations of these collagen fractions in the denervated muscle suggest indirectly that the loss of 'trophic influence due to denervation' results in modifications in the cross-links of the collagen fibril. Increase in glycoprotein level is in conformity with the findings of ANDREW and APPEL[8], who reported an increase in sialic acid content, a constituent of glycoproteins on denervation of a rat muscle. Variation in the hydroxy proline content in the present results demonstrates the occurrence of

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Table II. Variations in the hydroxyproline content of collagens in the normal and denervated cremaster muscle of bonnet monkey

Fraction	mg hydroxyproline/g collagen		Incidence of change on denervation
	Normal	Denervated	
Salt-soluble	146 ± 12	189 ± 8	Increase $p < 0.001$
Acid-soluble	128 ± 6	124 ± 9	No change $p > 0.01$
Alkali-soluble	138 ± 12	132 ± 16	No change $p > 0.01$
Insoluble	142 ± 16	181 ± 18	Increase $p < 0.001$

Values are $\bar{X}$ ± SD of 5 observations.

chemical modifications of the structure, since such modifications, even in the collagens of organisms subjected to environmental stress, have long been recognized[9]. Recently TRELSTAD[10] and HAY[11] have described 4 different molecular species of collagens which represent different structural gene products. It stands to reason therefore that the 'loss of trophic influence' in the cremaster muscle due to 60-day-chronic-transection of the nerve brings forth the quantitative and qualitative changes in the collagens, which imply the impairment of the action of specific structural genes[12].

Summary. Denervation of genitofemoralis in the bonnet monkey for 60 days resulted in a significant increase in neutral salt-soluble, alkali-soluble and insoluble collagens as well as glycoproteins. The hydroxyproline content of the salt-soluble and insoluble collagens in the muscle increased on denervation. These changes are discussed to imply the impairment of the action of specific structural genes.

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Mutagenic Effect of Ethionine on *Candida lipolytica*

Ethionine, the structural analogue of methionine, was first synthesized by DYER in 1938[1] and was shown to be detrimental to rats, and later by other workers, to many microorganisms at a level of about 2 mg per ml. Its effects, as so far known, is that it can compete with methionine for enzyme-binding sites and thereby interfere with methionine metabolism. Regulatory mutants of microorganisms have been obtained with resistance to ethionine which overproduce methionine[2-4]. During our attempts to isolate ethionine resistant strains of *Candida lipolytica*, we observed that ethionine itself can induce mutations in this organism to auxotrophy, morphological variation and possibly resistant to the analogue itself.

Table I. Effect of UV-irradiation on survival and ethionine resistance in *Candida lipolytica*

UV-dose (min)	Survivors (%)	Frequency of ethionine resistant strains ($\times 10^{-4}$)	
		3 days	6 days
0	100	0	0.178
2	100	0.355	0.550
4	6.980	0.822	1.031
6	0.021	—	—
8	0.003	6.950	8.130

Table II. Effect of ethionine on survival and mutagenesis in *Candida lipolytica*

Ethionine concentration (mg/ml)	Survivors (%)	Petite colonies ($\times 10^{-4}$)	Auxotrophs (%)
0	100	0	0
1	66.6	0.6	0
2	59.2	4.0	0
4	48.1	7.0	0
8	7.4	8.5	1.8
12	3.7	9.8	1.0
16	3.7	9.0	2.0

Ethionine mutagenesis in this case was suspected when UV-irradiation was employed to mutagenize the culture for isolation of ethionine-resistant strains. The non-irradiated control samples of the culture when plated out on ethionine began to show not only the morphological variations but also a significant number of ethionine-resistant colonies. The following procedure was adopted: 5 ml cell suspension of *C. lipolytica*, containing about 10^5 cells per ml from exponential growth phase, was irradiated with UV of 10 Ergs per mm^2 per sec for different periods of time ranging from zero (control) to 8 min in open petri dishes. After overnight refrigeration, the cultures were plated out on malt agar containing 10 mg DL-ethionine (Koch Light Labs. Ltd.) per ml. They were also separately plated out without ethionine for viable count. All the plates were incubated at 30 °C.

The inactivation of cells due to UV and the corresponding ethionine-resistant mutants among the survivor can be discerned from data in Table I. The ethionine plates were observed for colonies and data are presented for observations made on the 3rd and 6th days of incubation. The control without UV-irradiation showed no ethionine resistant colonies on the 3rd day, but on the 6th day they were seen at a frequency of 0.178×10^{-4}. In the irradiated samples, ethionine resistant colonies were observed on the 3rd day itself, but in all cases a significant increase in their frequency was recorded on the 6th day. All the ethionine plates, both UV-irradiated and non-irradiated showed morphological variants of wrinkled, petite and coloured colonies.

The UV-induced ethionine resistance is clearly evident by its proportionality to UV-dose (Table I). However, the emergence of ethionine resistant colonies in the case of non-irradiated control culture over prolonged incubation, as well as the occurrence of morphological variants, could not be satisfactorily explained. It was probable that these mutations were caused by ethionine itself after prolonged periods of contact with the cells.

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