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4. I am indebted to A. C. Giese, under whose direction this work was done, and to C. S. Pittendrigh for helpful discussions.
5. The contact was damped each minute by flexible contact arms. The male rhythm was checked later after some days in constant darkness (that is, red light) by turning off the alternators, and it was found to persist as before.
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New Metabolites of Serotonin in Carcinoid Urine

5-Hydroxyindoleacetic acid, the only metabolite of serotonin so far identified, represents less than 20 percent of an exogenous dose of serotonin (*1*). The reported presence of other unidentified indole derivatives in carcinoid urines (*2*) and rat liver perfusates (*3*), however, is indicative that metabolic reactions other than deamination occur. Suggestive evidence has also been presented that free serotonin is present in normal urine (*4*). Now it has been found that carcinoid urine is much more oxytocic than normal urine (personal observation) and it was thought that, if in this syndrome there was an increase in the excretion of the known metabolite, there would also be an increase in the unknown metabolites.

By an adaptation of the method described by Bumpus and Page (*4*), the indoles were extracted from 3 gal of carcinoid urine from one patient which contained 350 mg of 5-hydroxyindoleacetic acid as assayed by the method of Udenfriend, Titus, and Weissbach (*5*). Removal of excess urea and a partial fractionation of indoles was accomplished by using a cellulose column and a single phase solvent of *n*-propanol/ammonia.

Paper chromatography of the concentrated extracts and fractions revealed the presence of six indole derivatives. Five of these were identified by means of paper chromatography in three solvents, oxytocic activity, and fluorescent spectra (see Table 1). One of these proved to be the normally occurring urinary indican, but the other four—5-hydroxyindoleacetic acid, 5-hydroxyindoleaceturic acid, 5-hydroxytryptamine and N-acetyl 5-hydroxytryptamine—were evidently metabolites by serotonin. The 5-hydroxyindoleaceturic acid was further characterized by enzymic hydrolysis of an eluate with chymotrypsin, to yield 5-hydroxyindoleacetic acid and glycine.

Table 1. R_F values, oxytocic activity, and fluorescent spectra of metabolites of serotonin and the normally occurring urinary indican.

Metabolite	R_F in solvent*			Oxytocic† activity	Fluorescent spectra (mμ)‡		
	A	B	C		Activation (max.)	Fluorescent (max.)	*p*H
Serotonin creatinine sulphate	0.48	0.64	0.86	+++	295	540	2
5-Hydroxyindoleacetic acid	0.15	0.80	0.03		300	355	7
5-Hydroxyindoleaceturic acid	0.23	0.84					
N-acetyl serotonin	0.75	0.81	0.86	±	310	370	7
Indican	0.40	0.43	0.56		300	400	7

* Blue spots were obtained when sprayed with *p*-dimethylaminobenzaldehyde in 1.5*N* HCl. Solvent systems used: A, propan-1-ol saturated with ammonia; B, *n* butanol–acetic acid–water (4:1:5); C, ethyl methyl ketone–2*N* ammonia (2:1).
† Oxytocic activity was determined on an eoestrus rat uterus. Activity was antagonized by brom-lysergic acid diethylamide.
‡ Fluorescent spectra were determined with an Aminco Bowman spectrophotofluorometer.

The metabolism of endogenous serotonin in carcinoid patients therefore appears to be very similar to that of exogenous serotonin in experimental animals which we have studied (*6*). Autoradiographs obtained from urinary extracts of rats and rabbits given radioactive serotonin have shown the presence of the same four metabolites with the addition of two other minor metabolites. One of these has been identified as the glucuronide of serotonin since it gave a positive indole test but did not give a blue color with 2:6 dichloroquinonechloroimide, indicating that the hydroxyl group was not free. An eluate of this compound gave a positive naphthoresorcinol reaction, confirming that it was an ether glucuronide. Quantitative estimations of glucuronic acid and ethereal sulfate excretion after administration of serotonin have also shown that some conjugation does take place.

Oxidation of serotonin in vivo is a theoretical possibility, and it was thought that the other minor metabolite might represent the product of such a reaction, though so far no definite experimental confirmation has been obtained since it is present in such small quantities.

No evidence has been found in these experiments to suggest that methylation of serotonin might occur.

The normal metabolic fate of serotonin therefore appears to be (i) deamination to 5-hydroxyindoleacetic acid with (ii) some subsequent glycine conjugation to form the aceturic acid, (iii) N-acetylation, (iv) conjugation with glucuronic acid, (v) excretion unchanged and (vi) possible oxidation.

Preliminary studies have shown that, although great amounts of serotonin are metabolized by carcinoid patients, there appears to be no qualitative difference from the normal mode of metabolism.

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Spontaneous Changes in Corn Endosperm Tissue Cultures

Spontaneous changes in the characteristics of plant tissue cultures are known to occur from time to time. The best documented change is that which occurs in connection with the isolation of habituated tissues (*1*). Habituated tissues are independent of exogenous auxin, in contrast to the normal tissues from which they are derived. The latter tissues require external supplies of auxin for growth in vitro. Another change which has been observed to arise is the appearance of a purely parenchymatous tissue from woody tissue cultures (*2*). Reinert (*3*) and Torrey (*4*) have described irreversible changes from compact calli to cultures of very loose masses of cells from *Picea* and pea root callus, respectively. The latter changes, however, were as-

cribed to manipulations of the nutrient medium. In this report changes in the characteristics of corn endosperm tissue cultures are described which cannot be ascribed to manipulations of the nutrient medium.

Sternheimer (*5*) announced the establishment of tissue cultures of an anthocyanin-bearing strain of maize endosperm and a nonpigmented tissue derived from the same pigmented tissue. Mrs. Sternheimer was kind enough to supply me with cultures of these tissues in 1953. Unfortunately, these tissues were lost through accident and new cultures were subsequently isolated.

The endosperm was isolated from the maize variety Black Mexican Sweet according to the methods of Straus and LaRue (*6*). The culture medium and methods for determining changes in weight are described in the same paper.

Colorless tissues arise with a frequency of about 1 in 4000 cultures of pigmented tissue. The frequency is raised somewhat if old pigmented cultures which have ceased growth are transferred to fresh medium. The change from pigmented to nonpigmented tissue is not entirely irreversible. Occasional nonpigmented cultures show discrete, small spots of pigmentation. Attempts to isolate and grow these pigmented spots have consistently failed. This is probably due to their very small size (0.5 to 1.0 mm) and hence to their inability to grow when separated from the main mass of tissue. The pigmented spots never get very large and are soon outgrown by the colorless tissue.

The nonpigmented tissue (PI-C) has a growth rate very nearly double that of the pigmented tissue (PI). In 25 days PI-C shows a 475 percent increase in fresh weight and PI a 246 percent increase. Auxin (1 mg per liter of indoleacetic acid) inhibits the growth of both tissues, so it is doubtful that the increased growth rate of PI-C represents the same type of habituation as is found with other tissue cultures. If PI tissues were stimulated to grow more rapidly by addition of auxin, then habituation of the PI-C tissue would seem reasonable.

Extracts of PI-C prepared by disintegrating the tissues in a blender with sufficient 95 percent ethanol to give a final concentration of 80 percent ethanol, by evaporating at room temperature, and by taking up the residue in water and adding it to the nutrient medium stimulate the growth of both tissues in the presence of casein hydrolyzate (2 g/lit.). The increase in growth is small (10 percent) but persistent and highly reproducible. Extracts of PI tissue, on the other hand, are growth inhibiting under the same circumstances. These extracts depress growth by 18 to 38 percent. The results obtained with the PI extracts are not as quantitatively reproducible as those obtained with PI-C, but there is a persistent growth inhibition.

Another change has been observed in the PI tissues more recently. Early in 1958, several cultures of PI were found to have a bright red color rather than the deep purple normally found in the PI tissues. The red tissue (PI-R) has been subcultured six times at the time of writing and has persisted in producing the red pigment. No quantitative experiments have been performed to determine whether there is any difference in growth rate between PI and PI-R. However, visual observation does not indicate any great difference in the growth characteristics of the two tissues.

Absorption spectra of extracts prepared by steeping PI tissue in 95 percent ethanol containing 2 percent concentrated HCl (vol./vol.) to extract the pigments show two absorption maxima, one at 325 mμ and one at 530 to 540 mμ. The near-ultraviolet peak is characteristic of the flavone pigments, and the peak at 530 to 540 mμ is characteristic of some anthocyanins (*7*). Extracts of PI-R show a shift in both maxima to 340 mμ and 520 mμ. Preliminary purification of extracts of both PI and PI-R was attained by chromatography (see below) and subsequent elution of the major bands. Spectra taken of these purified pigments showed only the maxima in the longer wavelengths: 540 mμ for PI and 520 mμ for PI-R. Extracts of PI-C tissue show only the peak at 325 mμ. Extracts of CE clone I-C (*6*) which is derived from a corn endosperm that is normally unpigmented show no absorption peaks over the wavelengths tested (310 to 600 mμ). It appears, then, that pigments closely resembling anthocyanins are still synthesized by the PI-C tissues, but the ability to synthesize anthocyanin has been lost.

Extracts of the three tissues (PI, PI-C, and PI-R) were examined chromatographically. The extracts were prepared in the same manner as those used for the spectrophotometric observations. The crude extracts were evaporated at room temperature in an air stream, and the residue was repeatedly extracted with 95 percent ethanol. The ethanol solution was filtered and spotted on sheets of washed Whatman No. 1 filter paper. The sheets were equilibrated for 8 hours with the lower phase of *n*-butanol:acetic acid:water (4:1:5). The paper was then irrigated (descending) with the organic phase of the same mixture for 12 hours at a temperature of 30°C. Extracts of the three tissues were cochromatogrammed on the same sheet.

PI and PI-R tissues have mixtures consisting of at least three anthocyanins. The three pigments are common to both tissues. However, there was one spot representing the major pigment for each tissue. For PI, the main pigment spot had an R_f of 0.36; for PI-R the R_f of the main pigment spot was 0.47. Both tissues showed both pigment spots, but in PI the spot at 0.47 was very faint and the PI-R spot at 0.36 was also very faint. Both tissues revealed a barely discernible pigment spot at R_f 0.3. Partial hydrolysis of the major PI pigment with HCl and subsequent chromatography showed that the spot at R_f 0.47 was not the aglycone; the aglycone had an R_f of 0.62. The chromatographic evidence, together with the absorption data, seems to indicate quite strongly that PI-R tissue is synthesizing an anthocyanin quite different from that produced by the PI tissue. Unfortunately none of the pigments have been positively identified yet.

Examination of the chromatograms with ultraviolet light revealed fluorescing spots which were common to all three tissues.

The evidence seems to point to definite spontaneous changes occurring in the endosperm of Black Mexican Sweet cultured in vitro. The change to colorless in the tissue cultures seems to involve a lack of synthesis of anthocyanin from the precursor common to both the anthocyanins and flavonols. The absorption at 325 mμ seems to indicate that the precursors or flavonols are still synthesized by PI-C tissue.

Whether the changes described are due to gene mutations or to chromosomal abnormalities cannot at present be stated definitely. Chromosome smears of PI, PI-C, and PI-R tissues reveal the same types of aberrant nuclear and chromosomal behavior as that described for CE clone I-C (*8*). These are a high frequency of chromosome bridges, lagging chromosomes, regular and irregular polyploidy, hypoploidy and multinucleate cells. It would appear, then, that there are many opportunities for changes in the characteristics of the tissue to occur due to chromosomal abnormalities (*9*).

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