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Thermal Protection of Choline Chloride from Decomposition by Ionizing Radiation

Changes produced by ionizing radiations in target materials—for example, polymerization of vinyl monomers (*1*) and inactivation of enzymes (*2*)—are in general enhanced by temperature elevation. It appears, however, that choline chloride, which at room temperature is one of the most radiosensitive organic solids known (*3*), becomes markedly radiation-resistant at 150°C.

Studies have been carried out at our laboratory (*4*) on the effects of ionizing radiation on nerve tissue constituents, including the cholinesterase system (*5*). Choline, a substance essential to nerve conduction, has been shown (*3*) to decompose by a free radical chain mechanism to trimethylamine and acetaldehyde when it is subjected to ionizing radiation as the pure crystalline chloride. When exposed at room temperature to 2-Mev electrons, Co^{60} γ-rays, or C^{14} β-rays, the *G* values—that is, the number of molecules decomposed per 100 ev—were 20, 175, and 1250 respectively; at −196°C the compound was stable.

In an attempt to determine the energy of activation of the radiation decomposition of choline chloride, the crystalline compound was exposed to Co^{60} γ-rays at room temperature, 50°C, and 150°C. At each temperature, three Pyrex ampules of twice recrystallized choline chloride, dried under vacuum and nitrogen at 110°C for 2 hours and vacuum sealed, were exposed to the radiation for varying periods of time. Three Co^{60} sources were used, two of which (delivering 232,000 and 792,000 rep/hr) were maintained at room temperature; the third (572,000 rep/hr) had a normal operating temperature of 50°C and was equipped with a furnace for higher temperatures. The percentage of remaining choline in each of the irradiated samples and its control was determined by the reineckate method (*6*). The *G* values, which are listed in Table 1, were then calculated for each run from the obtained semilogarithmic relationship between the percentage of remaining choline and the radiation dose in rep.

Decreasing the radiation dose rate or increasing the temperature from 20° to 50°C resulted in higher yields of decomposition. However, at 150°, regardless of the radiation exposure (4.6 to 13.7×10^6 rep) only 9 to 13 percent of the choline decomposed. The nonirradiated control, which was also kept at 150°C, did not change in appearance. The irradiated samples, however, became brown, and a small amount of insoluble material formed.

In view of these changes, it was necessary to determine whether more than one compound was responsible for the very high choline recovery as indicated by the reineckate analyses. Cholinemethyl-C^{14} chloride was synthesized (*3*) and recrystallized twice. The product had a specific activity of 66.5 mμc/mg of choline chloride (*7*) (calculated, 62.5 mμc/mg). It was shown to be chromatographically pure (Whatman No. 1 paper and 4/1/1 *n*-butanol, concentrated HCl, and water, followed by autoradiography). The labeled choline was irradiated at 150°C in the same manner as the nonlabeled material. Once again, some brown and insoluble substances were formed.

Analysis of the soluble material by the reineckate procedure indicated that the choline recoveries following exposure to 1.4, 2.8, and 3.7×10^7 rep were 94, 94, and 93 percent, respectively (*8*). Solutions of the sample which had been irradiated for 64.5 hours at 150°C, and of the labeled control, which also had been held at 150°C for the same length of time, were chromatographed for 23 hours. Included on the same chromatogram were samples of C^{14}-labeled trimethylamine and nonheated, nonirradiated, labeled choline. The heated and nonheated control samples showed a single spot only. The irradiated sample activity was predominantly at the same R_f as the choline controls with only a faint trace (about 1 percent) at a slightly higher R_f. Apparently, therefore, no C^{14}-labeled compound other than choline had contributed to the color developed in the analytic procedure.

It might be assumed that the brown, irradiation-induced materials had acted as inhibitors of the free radical chain degradation of the remaining choline. If this were the case, one would expect that this type of inhibition should have been evident not only at 150°C but also, to some degree, at lower temperatures.

Lemmon (*3*) has suggested that the spatial arrangement of atoms of crystalline choline chloride may play an important role in the free radical chain degradation. One might speculate that thermal excitation at 150°C (in contrast to that at lower temperatures or to the excitation due to ionizing radiation per se) can disturb the arrangement sufficiently to prevent the chain reaction. Further studies, therefore, may help to determine the relationship of crystalline structure to free radical chain reactions in solids, as well as to establish the use of elevated temperatures to protect some labile materials during irradiation.

Table 1. Effect of temperature and dose rate on Co^{60} γ-ray decomposition of choline.

Co^{60} source (rep/hr)	Temp. of irradiation (°C)	Dose causing 50% decompn. (reps)	G values
792,000	18–20	2.9×10^7	143
232,000	20–25	1.0×10^7	415
572,000	50	0.8×10^7	520
572,000	150	*	

* Regardless of the dose—that is, from 0.5 to 3.7×10^7 rep.—approximately only 10 percent of the choline decomposed.

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8. Since the furnace in this Co^{60} source required approximately 15 minutes to either heat up or cool down from 150°C after the addition or removal of a sample, it is possible that the observed small choline losses were initiated in these periods.

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Segregation of Plasmagenes and the Determination Problem

Investigations on the willow-herb (*Epilobium*) have shown that the intra-individual segregation of plasmagenes is a basic character of cytoplasmic inheritance (*1*). During vegetative cell divisions the plasmagenes may be distributed accidentally. They may, however, enter more or less exclusively one of the daughter cells as well. In such a way differences of cells and characteristic patterns arise within the plant. Besides the cytoplasmic segregation occurring in

Epilobium, a more generally known example is the distribution of plastids in white-spotted plants. Struck by these phenomena, I have advanced the hypothesis that several processes of determination are caused essentially by an unequal distribution of plasmagenes during somatic development (*2*).

In this respect, the cytoplasmic alteration "irregulare" (*3*) offers special interest. "Irregulare" arises either after treatment by radioactive isotopes or spontaneously in certain interspecific hybrids which contain cytoplasm of the race "Essen" of *Epilobium hirsutum.* The "irregulare" character is transmitted nearly constantly through numerous generations only by the mother, even in presence of different nuclei foreign to the cytoplasm. During the ontogenetic development, especially during the development of the leaves, new cytoplasmic alterations are frequently given off by "irregulare" plants. It is in this way that "irregulare" leaf patterns originate. If a new lateral shoot is formed in such an anomalous area, maternal inheritance can be proved by crossing experiments. Therefore plasmonic [*plasmon* means the sum of all extranuclear genes (plasmagenes)] alterations must be involved.

One of the plasmonic alterations that arises frequently from "irregulare" or otherwise by treatment with radioactive isotopes is the alteration "rhytidiophyllum." The leaves or leaf patterns of the altered plants show a more or less strongly wrinkled lamina. By means of anatomical investigation, different causes of this wrinkling have been revealed in various "rhytidiophyllum" plants.

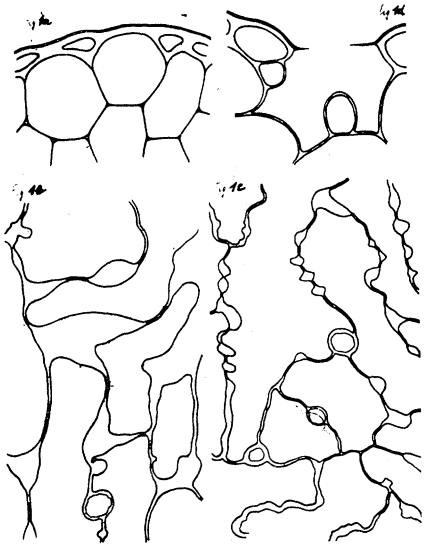

Fig. 1. Epidermis of the plasmonic alteration "rhytidiophyllum." (*a*) Cross-section of the epidermis with separated epidermal cells. Parenchyma cells slip into the interstices. (*b*) Surface view of the epidermis with warping cell walls. In the lower part, a pore has formed by bursting of the cuticle. (*c*) Similar view with cell walls warped like strings of pearls and with pores between the epidermal cells. The middle pore has an oblique position. (*d*) Cross-section through a pore with a torn cuticle.

Within a majority of "rhytidiophyllum" plants the division of epidermal cells soon stops. If this takes place at an early stage of development of the epidermis, the easily warping cell walls become expanded, and the cell bodies are separated. Parenchyma cells slip into the interstices, until they touch the cuticle. In many cases a cuticle covers these different cell types in a normal way. Occasionally it bursts and forms openings, which recall the pores of stomata (Fig. 1). If division of epidermal cells stops later, the development of stomata will be disturbed. Then a single guard cell may develop with a more or less normal slit at its side, or an air chamber below an undeveloped stoma mother cell. In a still later stage the stomata are stretched out into large apertures, below which the parenchyma cells lie open and shrink up. In extreme cases intercellular spaces between the epidermal cells become widened, or individual epidermal cells become torn. Then the epidermis covers the parenchyma only as a perforated net (Fig. 2). In all these cases the "rhytidiophyllum" character is produced by disharmonic development of two independent cell layers.

In some chimeras of "albomaculatum," in which cell division in the white parenchyma ceases early, the epidermis nevertheless continues dividing and lies in high folds. If individual white cells amid normal palisade parenchyma cannot divide, the white palisade cells are stretched out and assume a shape similar to those of the spongy parenchyma. Vice versa, normally dividing green spongy parenchyma cells amid white spongy parenchyma of white-spotted plants assume the shape of palisade cells.

During normal development, the typical shape of the two different parenchymas is essentially determined by their different rates of cell division. The origin of "rhytidiophyllum" disturbances agrees with these observations, and thus one may prove that they are caused by segregation of plasmagenes.

In a second type of "rhytidiophyllum," the epidermal cells do not grow horizontally, but vertically, to the leaf surface. New cell walls are built up periclinally (Fig. 3*a*). Such a multiseriate epidermis slightly resembles the epidermis of some xerophytes.

The third "rhytidiophyllum" type, and that of special interest, was found in relatively numerous plants. In these the cells of the mesophyll are transformed into cells of a new and complete epidermis. In the case of local "rhytidiophyl-

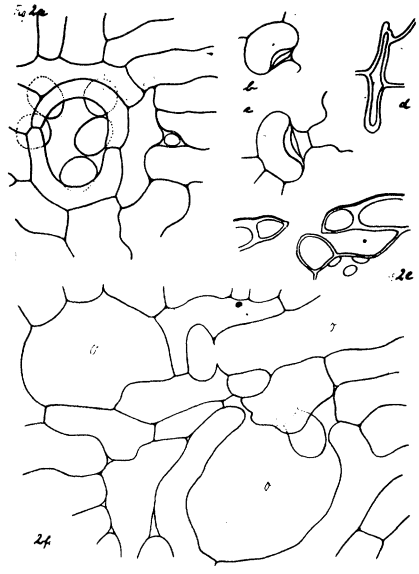

Fig. 2. Epidermis of "rhytidiophyllum." (*a*) Surface view of the upper epidermis of the leaf with one stoma and with intercellular spaces widened by the growing parenchyma. Below the open stoma, some cells of palisade parenchyma are discernible. (*b*) Disturbed stoma with only one guard cell beneath the slit. (*c*) Disturbed stoma; one of the guard cells has not developed. (*d*) Optical section through the air chamber below an undeveloped stoma mother cell. (*e*) Cross-section of a stretched stoma similar to that of *a.* (*f*) Epidermis stretched out to form a net; *o,* openings in the epidermis.

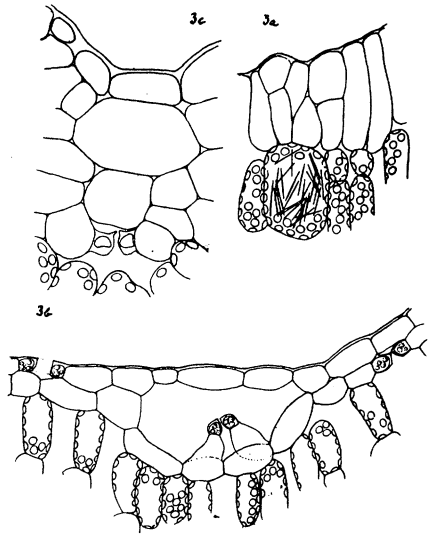

Fig. 3. Epidermis of "rhytidiophyllum." (*a*) Cross-section of the upper epidermis of the leaf; cells are lengthened and partially divided by periclinal walls. (*b*) Double epidermis, both layers with stomata. The guard cells of the inner stoma in the air chamber project as in some hygrophytes. (*c*) Multiseriate epidermis. In the uppermost layer no stoma were touched by the knife. In the innermost layer, which is transformed from the parenchyma, a normal stoma is above the central parenchyma.

lum" patterns, this transformation takes place within relatively late phases of leaf development. If there rises only one second epidermis, it will contain typical stomata and typical mother cells of hairs. In the interior of the leaf, however, these hairs do not develop further. In some cases this second epidermis is situated immediately below the normal one; in other cases it lines the much enlarged air chambers (Fig. 3*b*). In the first instance, the stomata of neither epidermis function. In the second case, the guard cells of the inner epidermis are lifted up under the influence of the high air moisture within the leaf. In most patterns with a double epidermis, one of the chlorenchyma layers is missing.

In some cases most layers of the spongy parenchyma have lost their normal chloroplasts and have changed into an epidermal tissue with intercellular spaces. Stomata were formed at the boundary between this and the normally green parenchyma amid the leaf (Fig. 3*c*). Other similar transformations of palisade parenchyma cannot be identified quite exactly as epidermal tissue because stomata are missing in them.

In the third type of "rhytidiophyllum" the following fact is of greatest interest: a plasmonic alteration, which can be proved as such, does not produce disturbances of normal development as in other cases of cytoplasmic inheritance, but a change of determination takes place. A meristematic tissue that should form chlorenchymatic cells develops instead into epidermal cells. This determination process differs from the normal formation of dermatogen only by the atypical moment of realization and by its abnormal localization. This difference, however, is caused by the time and locality of cytoplasmic segregation. If we start from the well-founded opinion that plasmonic segregation is produced by an unequal distribution of plasmagenes, we then logically arrive at the further conclusion that similar proceedings take place during the determination of the typical dermatogen as well, and that plasmagenes are distributed irregularly during the first cell divisions of the embryo (*4*), in which root and shoot and later on the layers of differentiated tissues are preformed.

Of course, cytoplasmic inheritance, as taking part in the processes of determination, is very difficult to prove by crossing experiments, and in many cases this task is impossible to solve at all. The aforementioned observations, however, show that the hypothesis of the significance of plasmagenes for determination possesses a high degree of probability. This hypothesis can be proved exactly by a more detailed investigation of the behavior of cytoplasm and its inheritance during ontogeny. One then will have to take into account that an analysis of intraindividual patterns is of the same importance for cytoplasmic inheritance as is an analysis of segregating crossings for chromosomal inheritance. Moreover, one should not forget that the fundamental principle of heredity means the identical reproduction and passing on of all genes during vegetative as well as during generative reproduction.

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Role of Fumarate in Formation of Stromata in "Vernalized" Ergot Fungus

It is well known that, for germination of *Claviceps purpurea,* an exposure of several weeks to cold, followed by a short period of exposure at higher temperature, is necessary. According to Kirchhoff (*1*), who studied this question in detail, the effect of cold seems to be similar to that of the vernalization of seeds. In studies of the respiration of germinating sclerotia and fully developed stromata of ergot of rye, I have found that fumarate plays a special role in this process.

The test ergot strain was Hungarian 12. An exposure to 0° to 3°C during 6 weeks was effective, causing 65 to 70 percent of the sclerotia to germinate after 3 weeks at 20°C on a double layer of wet filter paper in petri dishes. The sclerotia that had been treated as described were vacuum infiltrated for 1 hour with distilled water (control) or with $2 \times 10^{-2}M$ fumarate under 20 to 30 mm-Hg pressure. Infiltration with water does not influence the development of stromata, while infiltration with fumarate entirely inhibits the germination. This inhibition can be overcome to some extent by infiltration with $2 \times 10^{-2}M$ succinate. Succinate by itself has no effect on germination.

In order to gain a deeper insight into this question, conventional Warburg respirometers were used for the estimation of oxygen absorption (Q_{O_2}) of the sclerotia and stromata that had been infiltrated with the various compounds described in Table 1. Measurements were carried out on four occasions in triplicate. As shown in Table 1, the fumarate does not inhibit the respiration of sclerotia, while the oxygen consumption of stromata was strongly affected by it. Succinate added in concentrations equal to those of the fumarate is able to renew oxygen uptake to a considerable degree.

Table 1. Effect of fumarate and succinate on the respiration of sclerotia and stromata of ergot fungus.

Infiltration	Q_{O_2}		
	Sclerotia		Stromata
	Control	Cold treated	
Distilled water	25	27	346
Fumarate ($2 \times 10^{-2}M$)	22	25	20
Succinate ($2 \times 10^{-2}M$)	24	26	387
Fumarate + succinate	25	26	276

A consideration of these results has led to the following tentative conclusions and working hypothesis. The respiration of stromata follows a different pathway from that of the sclerotia. An explanation could be given for the inhibitory effect of fumarate on germination by the fact that, in the presence of fumarate, the respiration of stromata is inhibited. On the basis of the compensatory effect of succinate, it may be assumed that an unknown acid metabolism plays an important role in the organization of the stromata of ergot fungus. This is in agreement with the work of Cantino (*2*), who studied the relationship between cellular metabolism and morphogenesis in *Blastocladiella emersonii.*

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Conditioned Inhibition of Respiration and Heart Rate in the Goldfish

Conditioned inhibition of breathing rate and heart rate has been reported for various mammalian species, including man (*1*). Typically, the termination of a light, sound, or some other conditioned stimulus (CS) is repeatedly associated with noxious electric stimulation of some