The effect of freezing on the length and average epiphyseal width of 7 day old embryonic chick tibiae after 3 days' cultivation.

Treatment	Mean log length		Difference (d)
	Initial	Final	
Unfrozen . .	0·616	0·800	0·184
Frozen . . .	0·622	0·676	0·054

Standard error of d: 0·015, 8 D.F.

Treatment	Mean log epiphyseal width		Difference (d)
	Initial	Final	
Unfrozen . .	0·124	0·200	0·076
Frozen . . .	0·122	0·206	0·084

Standard error of d: 0·012, 8 D.F.

The measurements obtained from the camera lucida drawings made after 3 days' cultivation are summarized in the Table. The results demonstrate that although the frozen bones elongated to a significant degree ($t_{(8)} = 3{\cdot}60$, $0{\cdot}01 > P > 0{\cdot}001$), the extent of the elongation was much less than that observed on the unfrozen controls. The Table also shows that considerable and equal growth of the epiphyses occurred in both the frozen and unfrozen bones.

Histological examination of the explants showed that the majority of the rounded epiphyseal and flattened cells survived the freezing, although in most specimens some cells in the centre of the epiphysis were dead (Fig. 1). In contrast, however, a large number of the cells in the diaphysis were dead. A few were alive and hypertrophied, but they appeared very distorted (Fig. 2). The periosteum and the zone of periosteal ossification was normal. In some specimens, cultivated for 6 days after freezing, mitotic figures were found in the outgrowth of fibroblasts, the periosteum and among the chondrocytes of the epiphysis.

(2) The tibiae from six chick embryos (6 days' incubation) were employed. The two treatments were storage for 1 h at −79°C and subsequent culture after (*a*) freezing in normal saline, and (*b*) freezing in 15% glycerol saline.

After 3 days' cultivation all the bones frozen in normal saline were dead. All the bones frozen in glycerol saline, however, survived the freezing. The degree of elongation varied from specimen to specimen. Two of the bones doubled their length and remained normal in shape, while the others elongated to a lesser degree accompanied by some distortion. In all specimens the epiphyseal and flattened cells were normal in appearance and showed mitotic activity. No necrosis was seen in these regions. Only a few cells were dead in the diaphysis, the majority being hypertrophic. In some regions of the diaphysis, however, the matrix was abnormal in structure, although it stained metachromatically with toluidine blue.

IV. *Discussion.*—The results show that it is possible to freeze, and subsequently revive, all parts of the cartilaginous tibiae from 6 day old chick embryos, provided glycerol is present in the freezing medium. In contrast, only the epiphysis of the tibiae from 7 day old embryos survive to any extent. Thus, during a period of 24 h normal growth and differentiation the majority of the cells in the diaphysis become sensitive to the effects of freezing and are not protected by glycerol. At this stage of embryonic development the tibia undergoes rapid differentiation, the most spectacular change being the hypertrophy of the diaphyseal cells.

The causes of cellular damage on freezing, and the mechanism of the protective action of glycerol which is found with almost all tissues studied, has been analysed from a general point of view by LOVELOCK[5]. It is conceivable that the failure of glycerol to protect the diaphyseal cells is due to increased susceptibility to raised electrolyte concentrations or to the high glycerol concentrations. Changes in the permeability of the cell membranes may also be involved. Alternatively, the formation of matrix may retard the penetration of glycerol.

The results of freezing tibiae from 7 day old embryos is also of interest from a morphogenetic point of view, for the effect is to destroy differentially the zone of hypertrophic diaphyseal cells. In this zone, unlike the zones of epiphyseal and flattened cells, mitoses are rarely seen[6]. Thus, the fact that elongation of the bone is suppressed when the hypertrophic zone is destroyed demonstrates experimentally that the process of hypertrophy plays an important role in determining the shape of the developing cartilaginous rudiment.

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Zusammenfassung

Tibien von sechs Tage alten Hühnerembryonen überleben eine Abkühlung auf −79°C. Im Gegensatz dazu werden die hypertrophischen Zellen der Diaphyse von Tibien siebentägiger Embryonen durch Gefrieren weitgehend zerstört, während der Rest der Rudimente überlebt.

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Uracil Metabolism in *Neurospora crassa*

That a reductive pathway for pyrimidine catabolism exists was first suggest by FINK *et al.*[1]. Later, by tracer experiments *in vitro* and *in vivo*[2], by chromatography[3], and by enzyme studies[4], it was established beyond doubt that thymine or uracil may be degraded to β-amino acids via the corresponding dihydropyrimidines and β-ureido acids in animal tissues.

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DiCarlo *et al.*[5] first suggested that such a metabolic pattern is operative in microorganisms. Others[6] have presented evidence that the reductive pathway of uracil is essentially the same in bacteria as in animal tissue. Apparently, there is a delicately balanced system between the degradation of uracil and its utilization for nucleotides[7]. The reductive pathway for uracil degradation is shown in Figure 1.

Mitchell *et al.*[8] reported on the properties of four distinct mutants of *Neurospora* that require pyrimidines for growth. This paper extends the observations of one of these mutants, Pyr-1 (263), to new substrates. The new substrates were suggested by the work of Kornberg[9], in which he has shown that pyrimidines are synthesized according to the pathway shown in Figure 2. The growth properties of Pyr-1, and other pyrimidine mutants which will be described elsewhere, are compatible with this scheme. This paper also describes the compounds accumulated by Pyr-1, and by wild-type 73a. These data are presented as evidence that a reductive pathway for uracil degradation, similar to the one reported for other organisms, exists in *Neurospora*, and that this pathway may be reversed to support growth of the pyrimidine-requiring mutant, Pyr-1.

The minimal medium used in these experiments is described elsewhere[10]. The pyrimidine supplements are added on a micro-molar equivalent (*mM* eq.) basis. (The molecular weight of uracil, 100 rather than 112, serves as a base.) The growth measurements reported are made from dried (24 h at 60°C) mycelial pads harvested from 40 ml of liquid medium in 250 ml Erlenmeyer flasks. During the growth period, the cultures are incubated at 27°C, and replicas are harvested at 24 h intervals following inoculation.

Fig. 1.—Reductive Pathway of Uracil Metabolism.

$$\text{Uracil} \underset{\pm 2H}{\rightleftarrows} \text{dihydrouracil} \underset{\pm H_2O}{\rightleftarrows} \beta\text{-ureidopropionate} \begin{cases} \rightarrow \beta\text{-alanine} \\ \rightarrow NH_3 + CO_2 \end{cases}$$

After a 96 h incubation period, on 10 *mM* eq. of the various substrates, Pyr-1 yields (in mg mycelia, dry weight) the following: uridine 92 mg, cytidine 100 mg, uracil 62 mg, dihydrouracil 19 mg, β-ureidopropionic acid 21 mg. See Figure 3 for comparative growth rates. On cytosine, only a trace of growth is found, and thymidine, thymine, ureidosuccinic acid, dihydroorotic acid, and β-alanine fail to support any growth. All of the compounds in the uracil degradation pathway, except β-alanine, are capable of partially satisfying the pyrimidine requirement of Pyr-1. These data indicate that in the absence of sufficient uridine, the reductive pathway is reversed to function synthetically.

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Fig. 2.—The Pyrimidine Biosynthetic Pathway (cf. Kornberg[9])

$$\underset{\text{acid}}{\text{Ureidosuccinic}} \xrightarrow{\pm H_2O} \underset{\text{acid}}{\text{dihydroorotic}} \xrightarrow{\pm 2H} \underset{\text{acid}}{\text{orotic}} \xrightarrow[\text{phosphate}]{PRPP} \underset{\text{phosphate}}{\text{orotidine-5'}} \xrightarrow{-CO_2} \underset{\text{phosphate}}{\text{uridine-5'}}$$

According to the pathway of uridine-5'-phosphate synthesis, Figure 2, and to the growth data presented above, Pyr-1, is genetically blocked between dihydroorotic acid and orotic acid; it grows on the latter and all subsequent compounds in the pathway, but not on the former. The products accumulated in the mycelia of Pyr-1, however, do not support this view. Mycelial extracts for chromatography are prepared by the methods of Fincham and Boylen[11]. From these studies, it is clear that ureidosuccinic acid accumulates in the mycelia, but dihydroorotic acid does not accumulate in detectable quantities. Since ureidosuccinic acid is not detected in the medium, but only in the mycelia, it is probable that it is impermeable. The disparity between the growth data and the accumulation products, as to the exact location of the genetic block, might be due to the impermeability of dihydroorotic acid. At any rate, the genetic block is beween ureidosuccinic acid and orotic acid, see Figuree 2.

Evidence that the reductive pathway operates catabolically comes from analyses of wild-type. The accumulation products of aerated culture media are determined by first concentrating 1 l of medium, after 72 h of growth, to $^1/_{25}$–$^1/_{50}$ the original volume. Following filtration and precipitation of excess salts in the cold, the concentrate is chromatographed by the methods of

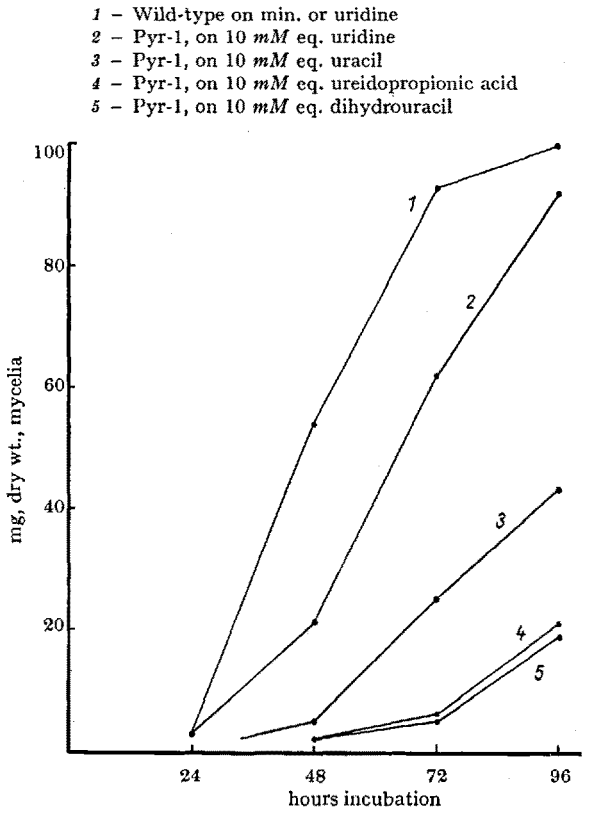

Fig. 3.—Comparative Growth Curves of Wild-type and Pyr-1 on Various Substrates

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FINK *et al.*[12]. When wild-type is cultured in minimal medium, the compounds of the reductive pathway, dihydrouracil and β-ureidopropionic acid, cannot be detected in the medium, but when grown in excess uridine these compounds accumulate. It is conjectured that during growth the uridine synthesized is used for nucleotides, but with excess uridine present, uracil arises from nucleotide degradation, and, after accumulating in sufficient quantity, is further degraded to β-alanine, NH_3 and CO_2.

There is also evidence of a uracil pool in *Neurospora*. Wild-type, cultured in 10 *mM* eq. of uracil, consumes approximately 70% of the uracil during the first 96 h of incubation according to spectral analyses. It is not known at present what proportion of this is degraded and what proportion is used for nucleotide synthesis, but, when grown in minimal medium, wild-type accumulates approximately 0·1 *mM* eq. of uracil without any detectable accumulation of dihydrouracil and β-ureidoproponic acid. That the accumulated product is uracil can be verified by cross-feeding experiments. It has been shown with rat liver slices[13] that the uracil → dihydrouracil reaction is the rate-limiting step of the degradation pathway; if this is so in *Neurospora*, as our preliminary evidence indicates, it would explain the existence of a uracil pool.

This work will be published in fuller detail elsewhere.

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Zusammenfassung

Der Pyrimidinbedarf der *Neurospora crassa*-Mutante Pyr-1 (263) kann durch alle beim reduktiven Abbau des Uracils durchlaufenen Zwischenprodukte, mit Ausnahme des β-Alanins, zum Teil befriedigt werden, nämlich durch β-Ureidopropionsäure, Dihydrouracil und Uracil. Wildwachsende *Neurospora* speichert nach Zulage von Uridin in der Nährlösung β-Ureidopropionsäure und Dihydrouracil, aber nicht bei Abwesenheit von Uridin. Aus diesen Feststellungen folgt, dass der reduktive Umbau des Uracils je nach den Konzentrationsverschiebungen in der Nährlösung im aufbauenden oder im abbauenden Sinne verlaufen kann.

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Variations in the Glycosidic Pattern of Anthocyanins

Part II[1]

A study of the glycosidic nature of the anthocyanin plant pigments is being carried out as part of an investigation of the role of glycosidation in anthocyanin biosynthesis. The discovery of a new type of glycoside, in which the anthocyanidin has two sugar residues in the 3-position and one in the 5-position, has been reported earlier[2]. Other novel types of anthocyanidin glycosides have now been found in a variety of plant material. Most progress has been made with glycosides of pelargonidin (3,5,7,4′-tetrahydroxyflavylium chloride) since it is possible in most cases to determine the position of the sugar residues without recourse to detailed chemical analysis. For example, pelargonidin glycosides which have a sugar residue in the 5-position display a characteristic yellow fluorescence in ultraviolet light[3].

The method of determining the position and nature of the sugar residues of anthocyanins employing the techniques of paper partition chromatography have been outlined elsewhere[4]. It consists of hydrolysing a solution of the anthocyanin, carefully purified by repeated chromatography, and identifying the aglycone and sugars by standard procedures. The number and position of the sugar residues is then obtained by controlled acid hydrolysis of the anthocyanin and examining all the simpler glycosides produced as intermediates. The comparison of R_f values with known pigments in a variety of solvent systems is also necessary for identifying new compounds. Since our earlier reports[5], one modification has had to be introduced into the method, since it was found that arabinose is produced as an artifact during the purification of anthocyanins on Whatman No. 3 paper if solvent mixtures containing hydrochloric acid are used. The presence of this acid was considered necessary for preventing the anthocyanin fading during chromatography. This difficulty has now been overcome by replacing the hydrochloric by acetic acid, and by washing the sheets of filter paper prior to their use with dilute acetic acid. As a result, it has been necessary to revise the provisional structures of some pigments described earlier as containing arabinose. Thus, the acylated pelargonidin derivative present in *Solanum phureja* is the 3-rhamnoglucosido-5-monoglucoside and the unusual cyanidin glycoside present in the stems of *Streptocarpus spp.*, in elderberries and in the leaves of *Begonia spp.* is cyanidin-3-xyloglucoside. In the same way, the cyanidin derivative of *Dahlia variabilis*, described recently by NORDSTRÖM[6] as the 3-glucosido-5-arabinoside, must be the 3:5-diglucoside, since this author based his identification on chromatographic methods using solvents containing mineral acid.

In all, some nine chromatographically distinct glycosides of pelargonidin have been examined, the well characterised 3-monoglucoside (callistephin) and 3:5-diglucoside (pelargonin) being available for comparison. Variation due to acylation was eliminated by subjecting pigments containing acyl groups to alkaline hydrolysis before further examination. Some of the nine glycosides fall into the 'classes' described by the ROBINSONS[7]. The majority of 3-monosides examined are identical with callistephin, but there is evidence that pelargonidin-3-monogalactoside occurs in trace amounts with cyanidin-3-monogalactoside in the leaves of the copper beech, *Fagus sylvatica*. The 3-monoglucoside and 3-monoga-

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