method is suitable for determining the heat of reaction per hydrogen bond between oligonucleotides and polymers. Some complexity is introduced in this case by the apparent formation of a triple-stranded structure (*11*) between one 5′ pApApA molecule and two polyU strands in the gel.

The degree of cross linking is important in the use of gels for studies of complementary relationships between nucleic acids. A tightly cross-linked gel retards the diffusion of nucleic acid strands into position for hydrogen bond formation. In addition, the presence of an interstrand cross link certainly would prevent hydrogen bonding in its immediate neighborhood and might have longer range effects. It was observed that one sample of polyU dried on Geon 101 beads and exposed to about 5×10^6 ergs/cm^2 of radiation at 254.7 mμ had a very much reduced capacity to hydrogen bond with even so small a molecule as pApApA. This was perhaps, in part, due to ultraviolet induced hydration of the uracil residues and other possible chemical effects. Opposed to the requirements for a low degree of cross linking is the need to prevent the polymer from going into solution at high temperatures where some thermal strand cissions will occur. The gels should therefore be made from as high molecular weight material as possible and irradiated only enough to provide stability. One solution to these conflicting requirements currently being tested is to form an extremely tightly cross-linked gel of polyU or a DNA unrelated to the nucleic acids otherwise participating in the experiment (*13*). The nucleic acid of interest then can be stably bound to this matrix with minimal irradiation and a small number of cross links.

The nature of the interstrand cross links which give rise to insoluble nucleic acid gels after ultraviolet irradiation is not yet fully understood. It has been clearly demonstrated that thymine dimers are induced by ultraviolet irradiation in frozen solution (*14, 15*) and that they occur in irradiated DNA (*16, 17*). Insoluble polyU gels have been previously reported (*11*) and evidence for a uracil dimer has been published (*17*). The formation of polyC gels by ultraviolet irradiation has not been previously reported. The irradiation of cytosine or cytosine C^{14}-labeled DNA in solution appear to give rise to a uracil dimer rather than a cytosine dimer (*17*). However, the irradiation doses were much higher than those used here and the product might be different with the high concentrations of cytosine residues in the dried polyC films. Some evidence has been presented for the existence of mixed dimers of uridine and thymidine (*17*). The conclusion can hardly be avoided as previously suggested (*15*), that dimers can form between any pair of pyrimidines. This would account for the relative ease with which insoluble gels of naturally occurring nucleic acids can be induced by ultraviolet irradiation It is very unlikely that purine-purine dimers play a part in the process. However, there is no evidence for or against purine-pyrimidine dimers. It remains to be determined which of the possible dimers are important in a particular situation.

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30 September 1963

Lactate Dehydrogenase Isozymes: Lability at Low Temperature

Abstract. *A change in the lactate dehydrogenase isozyme pattern can arise when human tissue homogenates are kept at −20°C. This change is most pronounced in a homogenate with a pH of about 7.9 in barbital buffer and is not seen when nicotinamide adeninedinucleotide is added to the homogenates before freezing, nor when the homogenates are kept at room temperature, nor when the tissue alone is kept at −20°C.*

Although differences in heat lability of the lactate dehydrogenase (LDH) isozymes (*1*), have been reported, little information is available on the influence of low temperatures on the stability of LDH.

In our laboratory, LDH isozymes of human tissues were studied with the aid of electrophoresis in agar gel on microscope slides (*2*). Tissue homogenates were prepared by grinding the fresh tissues in a Potter-Elvehjem glass homogenizer with about 1 g of tissue to 4 ml of barbital buffer, *p*H 8.4, ionic strength 0.1, and then by centrifuging for 30 minutes at 3000*g*. The actual *p*H of the homogenates thus obtained is about 7.9. Prior to electrophoresis the homogenates were diluted with buffer to a convenient LDH activity. Five active LDH fractions are found in most tissue extracts, in different relative proportions depending on the tissue.

Total loss of activity in the slower moving isozymes LDH-4 and LDH-5 occurred when the concentrated tissue homogenates were kept overnight at −20°C. This loss of activity was not seen when the extracts were kept at room temperature, nor when the tissue itself was kept at −20°C. When the isozyme pattern of a tissue homogenate kept at room temperature (Fig. 1*a*) was compared with that of the same homogenate kept at −20°C (Fig. 1*b*), (i) the activity of LDH-1, the fastest moving anodal fraction, is the same in both samples; (ii) the activity of LDH-2 is the same or probably a little less in the −20°C sample; (iii) the activity

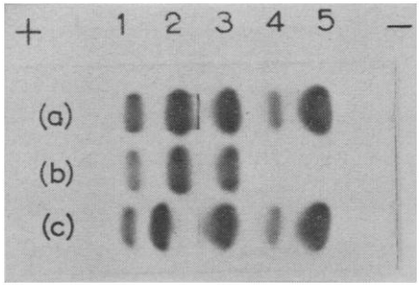

Fig. 1. LDH isozyme patterns of human skeletal muscle (homogenate at *p*H 7.9 in barbital buffer). (*a*). Homogenate kept overnight at room temperature (the pattern is unchanged as compared with that of the freshly prepared homogenate). (*b*). Same homogenate kept overnight at −20°C with no NAD added. (*c*). Same homogenate with 10 mg NAD per ml added and kept overnight at −20°C. It can be seen from these patterns that, apart from the stabilizing effect on LDH-4 and LDH-5, an additional, and as yet unexplained effect of NAD on the electrophoretic mobility of LDH-1 and LDH-2 occurs.

of LDH-3 in the −20°C sample is about half of the activity of LDH-3 in the room temperature sample; (iv) the activity of LDH-4 and LDH-5 in the −20°C sample is completely lost.

In order to determine the influence of *p*H and type of buffer on the observed change in isozyme pattern further experiments were carried out with homogenates in barbital buffer (actual *p*H 7.0), tris-HCl buffer (actual *p*H 7.9 and 7.0) and phosphate buffer (actual *p*H 7.4). In barbital buffer *p*H 7.0 the disappearance of LDH-4 and LDH-5 at −20°C was not observed, at least not within 24 hours. If, however, homogenates were diluted 1 : 5 with buffer before freezing, the change in pattern at this *p*H was exactly the same as at *p*H 7.9. In homogenates made with tris buffer, total loss of activity in the labile fractions occurred only at *p*H 7.9, and only after keeping the extracts for 96 hours at −20°C. In phosphate buffer at *p*H 7.4 no disappearance of fractions was seen. Although complete inactivation of LDH-4 and LDH-5 within 24 hours is thus found only in barbital buffer, *p*H 7.9, less pronounced changes in isozyme pattern were nevertheless frequently observed at −20°C in homogenates made with other buffers, including phosphate buffer *p*H 7.4. These less marked changes always concern the activity of LDH-4 and LDH-5.

Thus, faulty results can be produced in establishing the isozyme pattern of tissues, especially when cell destruction is effected through repeated alternate freezing and thawing of the cell suspension. Apart from its practical consequence, the behavior of the LDH isozymes at low temperatures is of considerable theoretical interest, and an explanation could contribute to the general concept of the chemical structure of LDH isozymes. The change in isozyme pattern during low temperature exposure, particularly the inactivation of the slower moving fractions, can be prevented in every instance by adding nicotinamide adenine dinucleotide (NAD) to the tissue extracts. No loss of activity occurs when 10 mg of (neutralized) NAD per milliliter of extract is added before freezing (Fig. 1*c*).

A possible explanation of the protective effect of addition of NAD upon the inactivation of LDH-4 and LDH-5 can be given in the terms of the concepts of Markert *et al.* (*3*) and Fritz *et al.* (*4*). According to Markert a molecule of LDH is composed of four subunits. These subunits are separable into two types, A and B, and combination of both types in some manner in groups of four, yields five different molecular species (A^0B^4, A^1B^3, A^2B^2, A^3B^1, and A^4B^0), thus accounting for the existence of five isozymes. Recently, Markert (*5*) has shown that dissociation of LDH-1 (B-subunits) and LDH-5 (A-subunits) in a mixture, followed by recombination of the subunits at random, indeed yields all five isozymes in vitro. Most interesting is the fact that in this dissociation and recombination experiment, Markert froze the mixture overnight. Fritz *et al.* (*4*) made the assumption that each monomeric unit has a molecule of NAD attached to it, and found evidence for the supposition that under certain conditions the NAD molecules attached to the A-subunits can be more easily removed than those attached to the B-subunits.

Our hypothesis is, that during the process of homogenization every A-subunit loses its NAD molecule, leaving the active sulfhydryl group of these subunits unprotected against inactivation. If exposure to low temperature can inactivate the enzyme activity of molecules consisting of subunits without attached NAD, this can indeed explain a change in the isozyme pattern (Fig. 2). Assuming that the subunits are inactive by themselves it can be expected that a molecule consisting of BN–A*–A*–A* (A* = inactivated A-subunit) is inactive as well, instead of being 25 percent active. The activity of molecules consisting of more than one active subunit is apparently proportional to the number of active subunits. Exact quantitative relations are not known. That NAD protects against cold inactivation is now understandable. The excess of NAD enables the A-subunits to pick up new molecules of NAD, and this protects them against inactivation.

To prove the basic assumptions, experiments were carried out with the addition of reduced glutathione (GSH) to the homogenates. It was assumed that the presence of GSH could prevent the inactivation of LDH-4 and LDH-5 at low temperature and that the activity lost from LDH-4 and LDH-5 after low temperature exposure could be restored by addition of GSH. Both assumptions proved to be correct. Addition of 10 mg (neutralized) GSH per milliliter to a homogenate before freezing protects the activity of the labile fractions. When, prior to electrophoresis, the same amount of GSH is added to a homogenate which has been kept at −20°C, the activity in LDH-4 and LDH-5 is, at least partly, restored and both bands reappear.

Reactivation and protection against cold inactivation of LDH-4 and LDH-5 by GSH is in accordance with the hypothesis proposed. In human serum, the loss of activity in the labile fractions during low temperature is less rapid than in most tissue homogenates. Complete inactivation of LDH-4 and LDH-5 in serum kept at −20°C occurs only after 1 or 2 weeks. At first sight, this is not in accordance with the hypothesis proposed. In serum, no NAD is present and on this account one would expect a greater

LDH-1 : BN–BN–BN–BN		BN–BN–BN–BN		BN–BN–BN–BN (100% active)
LDH-2 : BN–BN–BN–AN	homogeni-	BN–BN–BN–A	low tem-	BN–BN–BN–A* (75% active)
LDH-3 : BN–BN–AN–AN	⟶	BN–BN–A–A	⟶	BN–BN–A*–A* (50% active)
LDH-4 : BN–AN–AN–AN	zation	BN–A–A–A	perature	BN–A*–A*–A* (inactive)
LDH-5 : AN–AN–AN–AN		A–A–A–A		A*–A*–A*–A* (inactive)

B = LDH-1 type subunit N = NAD molecule
A = LDH-5 type subunit A* = inactivated A

Fig. 2. Proposed explanation for the change in isozyme pattern at low temperature.

sensitivity to cold inactivation in this medium. Probably the high content of protein (SH-groups) in serum exerts a protective influence on the enzyme molecules. Nevertheless, LDH activity in serum is better preserved when kept at room temperature instead of in the refrigerator, especially in the case of a high concentration of LDH-5.

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9 September 1963

Actinomycin D: Specific Inhibitory Effects on the Explanted Chick Embryo

Abstract. *Chick embryos containing 11 to 13 somites were cultured for 48 hours on media containing various amounts of the antibiotic actinomycin D. Morphological observations as well as quantitative determinations of protein nitrogen, DNA, and RNA indicate that the antibiotic specifically inhibits the growth and development of the embryonic axis posterior to the 12th somite.*

Injection of 0.125 μg of the antibiotic actinomycin D into the yolk sac of chick embryos during the second day of incubation causes cell death in the undifferentiated nodal mass and in the unsegmented somite mesoderm (*1*). Subsequent degeneration in these regions interferes with normal development of the embryonic axis (*2*). Depending on the extent of interference, abnormal embryos so produced have been classified as either "trunkless" or "rumpless." Trunkless embryos are (i) those which consist solely of a head, neck, and heart region, (ii) those which lack portions of the trunk, including the hind appendages, or (iii) those which have constricted trunks and abnormal limbs. Rumpless embryos lack the pygostyle, free caudal vertebrae, and, usually, varying numbers of the synsacral vertebrae. The extent of the axial defect resulting from treatment with the antibiotic is related to the developmental stage of the embryo at the time of treatment as well as to the dosage employed. Trunklessness is more common after treatment at 32 hours' incubation with 0.0625 μg of the antibiotic, and rumplessness is more common after treatment at 48 hours' incubation. The frequency of trunklessness obtained after treatment at 48 hours can be increased, however, by increasing the dosage of antibiotic.

We have undertaken to elucidate the mechanism of the deleterious action of actinomycin D in the chick embryo. Because there is considerable variation in developmental stages among embryos of any given incubation age, and because the response of an embryo to the antibiotic varies with the developmental stage, we are using chick embryos cultured in vitro. The in vitro explantation technique allows for standardization of the experimental material at specific developmental stages, assures uniform distribution in the medium of the substance to be tested, and facilitates use of quantitative procedures for analyzing the response. Having completed the preliminary phases of these studies, we now present evidence inditating (i) that actinomycin D interferes specifically with the development of the axial skeleton of the chick embryo cultured in vitro and (ii) that this interference is restricted to those levels of the embryonic axis which are normally elaborated from the unsegmented somite mesoderm and undifferentiated nodal mass subsequent to the time the embryos are exposed to the antibiotic.

Fertile White Leghorn eggs from a local commercial source were incubated in a forced-draft incubator at 37.5°C for approximately 40 hours, and embryos containing 11 to 13 somites were selected for explantation according to the procedures of Klein *et al.* (*3*). Embryos were explanted onto a concentrated whole egg homogenate medium (*4*) to which various amounts of actinomycin D had previously been added. Control embryos were cultivated on the medium alone. For the first 24 hours, all cultures were maintained under an atmosphere of 25 percent oxygen and 75 percent air. For the next 24 hours the atmosphere was changed to 95 percent oxygen and 5 percent carbon dioxide (*5*).

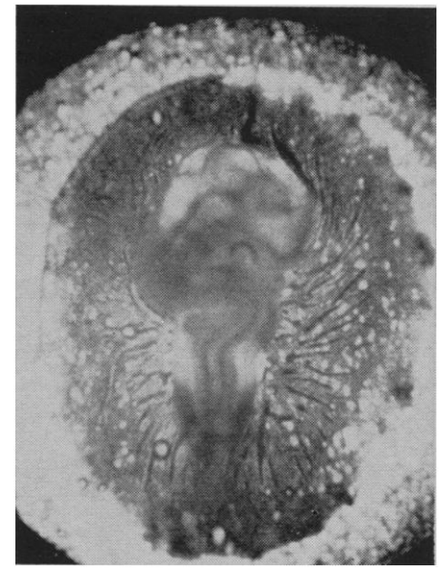

Fig. 1. Control embryo explanted when it had 11 to 13 somites and then cultured for 48 hours in vitro.

Under these conditions 57 of 58 control embryos survived 48 hours and showed well-developed heads, tails, and limb buds (Fig. 1). All embryos (55) exposed to 0.125 μg of actinomycin D, 52 of 57 embryos exposed to 0.25 μg, and 28 of 57 embryos exposed to 0.5 μg survived the culture period. The response to 0.125 μg of the antibiotic was variable, but virtually all embryos exposed to higher doses showed some

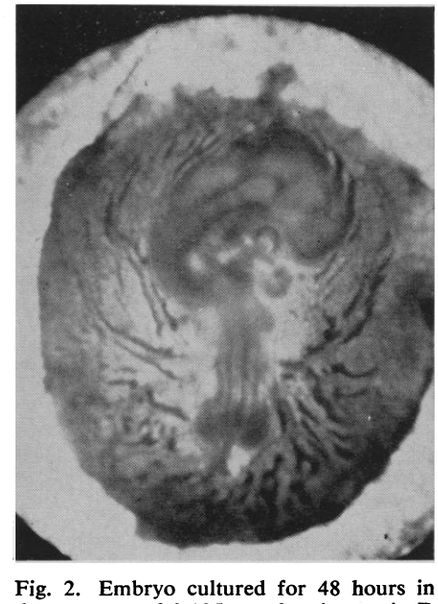

Fig. 2. Embryo cultured for 48 hours in the presence of 0.125 μg of actinomycin D per milliliter.