excitation spectra, taken on the same instrument with identical bandwidths, is not evident for any base and may be taken as an implication that there is a variation in the emission yield as a function of excitation energy (*5*). Other examples of such behavior have been reported for the purines and pyrimidines under different conditions. At 170°K in EG:H_2O (70:30, by volume) the relative quantum yields of adenine fluorescence excited at 4.00, 3.73, and 3.57 μm^{-1} are 0.6, 1.0, and 1.9 (*10*), as compared with our values at room temperature and in aqueous solution of 0.54, 1.0, and 2.7. Excitation spectra of guanine (*11*) at acid and alkaline *p*H's, of adenine (*12*) in acid, and of thymine (*13*) in alkali also exhibit a shift relative to absorption similar to our results. Processes that may explain this type of shift include the existence of two tautomeric structures in the first absorption band, only one of which is fluorescent (*5, 10, 12*), the changing efficiency of radiationless deactivation processes as a function of excitation energy (*5*), and emission from an n-π^* or π-π^* state hidden in the red edge of the absorption band (*5, 14*).

The emission quantum yields (Table 1), calculated relative to a PPO (2,5-diphenyloxazole) yield in nitrogen-flushed cyclohexane assumed to be 1.00 (*15*), are sufficiently low to have escaped previous detection. At room temperature the purines fluoresce at least three times more intensely than pyrimidines, whereas at 77°K cytosine fluorescence is reported (*9*) to be as efficient as that of the purines, and thymine fluorescence (*8*) has more than twice the quantum yield of the purines. These changes in the relative order of emission yields as a function of temperature (for example, from 77° to 300°K the guanine yield is reduced 200-fold, but the thymine yield is reduced over 2000-fold) further emphasize the uncertainties in the direct application of low-temperature results to room temperature conditions.

The low observed quantum yields imply correspondingly short singlet lifetimes, $\sim 10^{-12}$ second. This short singlet lifetime has been interpreted as making singlet energy transfer in DNA improbable (*4*) [the estimated transfer rate is 10^{12} sec^{-1} under the most favorable conditions (*16*)]. The possibility, however, of emission arising from a tautomer or excited state hidden in the first absorption band makes the true quantum yields and the oscillator strengths of the fluorescing species somewhat uncertain. Calculated singlet lifetimes are all $\sim 10^{-12}$ second or less [Table 1, column (a)] if it is assumed that the entire low-energy absorption band for each base is responsible for emission. If, however, the fluorescent oscillator is assumed to be the fluorescence excitation fitted to the low-energy absorption band [which may give an estimate of that portion of the total absorption actually responsible for fluorescence (*10*)], all lifetimes are significantly increased [Table 1, column (b)]. Singlet lifetimes at room temperature calculated directly from the quantum yield and the absorption spectrum must therefore be considered a lower limit. In addition, the slightly broader emission spectra reported for all the bases at 300°K relative to 77°K may mean a different overlap integral for singlet transfer. The role of singlet energy transfer at room temperature must remain an open question.

The O-O′ energies (Table 1) are in the order adenine ~ uracil > cytosine > thymine > guanine. Although different from the order for the nucleotides at 77°K (*16*), particularly in the position of guanine, our values are very similar to those determined (*17*) at an intermediate temperature of 195°K for adenine (3.56 μm^{-1}) and guanine (3.32 μm^{-1}) (*17*). Although room-temperature data on the nucleotides would be preferred for comparison, the results presented here may mean that the relative excited state energies of the bases are sufficiently altered by temperature to affect the direction of energy transfer.

In view of the significant differences in excited singlet properties between 77° and 300°K, extrapolation from low-temperature measurements to processes occurring in DNA under biological conditions may require careful reevaluation. Extension of the technique reported here to an investigation of the fluorescent properties of the nucleotides, excimer formation (*18*) in the dinucleotides, and the fluorescence behavior of DNA itself is now feasible.

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17. Estimated from the overlap of absorption and emission spectra given in (*7*).
18. An excimer (*1*) may be thought of as the excited complex of two identical molecules, that is, an excited-state dimer.
19. Supported by the Atomic Energy Commission (Division of Biology and Medicine); this report constitutes AEC report RLO-2014-11. We thank Dr. I. Berlman for generously furnishing large-scale spectra of fluorescent standards and the Pacific Northwest Water Laboratory for the use of their Turner model 210 spectrofluorimeter.

24 September 1970

Subunit Structure of Aldolase

Abstract. *A new crystal form of rabbit muscle aldolase shows that the molecule has 222 symmetry to at least 4-angstrom resolution, and hence that the gross conformation of the four subunits is the same. Comparison of the new form with a previously reported form establishes the number of molecules per unit cell,* n, *in the older form. For an independent check, the "crystal-volume and protein-content method" was developed to determine* n *without directly measuring the water content of the crystals.*

Aldolase from rabbit muscle is composed of four subunits, each having a molecular mass of about 40,000 daltons (*1*). Two crystal forms of this enzyme have been reported (*2, 3*): form I (Table 1) is a hexagonal form that is

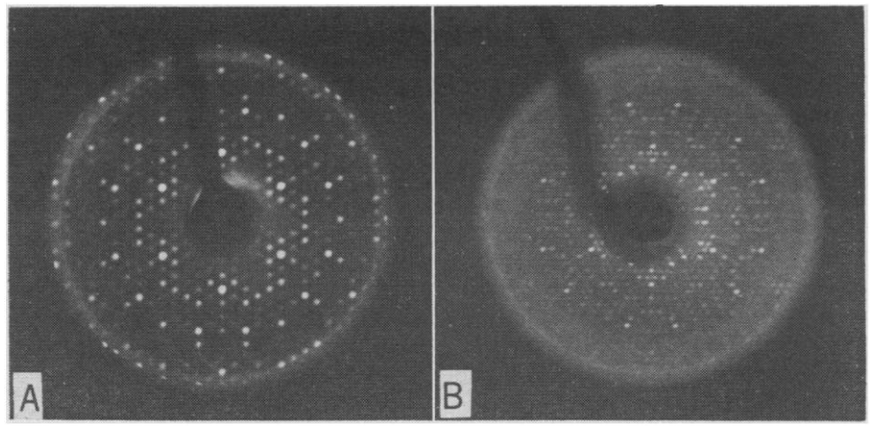

Fig. 1. (A) The *hki*0 section of the diffraction pattern for form III, with a^*-axis vertical. (B) The same section for form I with the a^*-axis 30° away from the vertical direction.

relatively unstable to x-radiation, and form II is monoclinic with the large asymmetric unit of one entire molecule. We have found a new modification, form III (Table 1) that exhibits a well-ordered diffraction pattern to a resolution of at least 3 Å, is quite stable to radiation, and has the smallest possible asymmetric unit, namely one enzyme subunit. The diffraction pattern of this form shows that the four subunits are arranged about three mutually perpendicular twofold axes, and, thus, that the gross conformation of all subunits is the same.

When several crystal forms of an enzyme are known, such as forms I, II, and III of aldolase, information on n, the number of molecules per unit cell in one form, restricts the possible values of n in the other forms. We have used this "method of multiple crystal forms" to establish that in form I n is nine. The value nine is also indicated by another procedure, the "crystal-volume and protein-content method," described below.

Aldolase (Boehringer-Mannheim) was dialyzed against 0.02*M* potassium phosphate buffer, *p*H 6.0, containing 1 m*M* glutathione and 0.1 m*M* ethylenediaminetetraacetic acid, and then concentrated with Lyphogel (Gelman Instrument Co.) to a protein concentration of 10 mg/ml. After the concentrate was centrifuged at 10,000 rev/min for half an hour, ammonium sulfate was added to a concentration of 2.3 mole/liter of this salt. Crystals grew in 2 to 6 weeks. All batches of crystals contained form I and about 10 percent of the batches also contained small crystals of form III. Attempts to find conditions that increase the incidence of form III were unsuccessful. Both forms have the external morphology of hexagonal bipyramids, and both possess aldolase activity. Activity was assayed by a coupled enzyme system, that employed glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8) and triosephosphate isomerase (E.C. 5.3.1.1). The oxidation of reduced nicotinamide adenine dinucleotide at 340 nm was monitored. Densities of the crystals were measured by flotation (forms I and III) and by the gradient-column method (form I).

The *hki*0 section of the diffraction pattern of form III is shown in Fig. 1A. We established that the space group is $P6_222$ (or its enantiomorph, $P6_422$) from the sixfold symmetry and mirror planes exhibited by both this and the *hkil* section, and from the absence of all reflection along the 000*l* row of the diffraction pattern except for those with indices of the form $1 = 3m$, m being an integer.

The symmetry of space group $P6_222$ restricts the number of molecules per unit cell to three, when taken together with our observed cell dimensions and density for form III, and with the molecular weight of aldolase. This can be shown from (*4*):

$$X_P = \frac{Mn}{N\rho V} \qquad (1)$$

where n is the number of molecules of molecular weight M in a unit cell of volume V, ρ is the crystal density, N is Avogadro's number, and X_P is the weight fraction of protein in the wet crystal. In space group $P6_222$, n is restricted to multiples of three. If n is initially assumed to be six, and M is taken as the generally accepted value of 160,000 daltons, X_P is calculated to be 0.98. This is an impossibly high value for a wet protein crystal, so that the number of molecules per cell in this form must be three.

The symmetry of the aldolase molecule can be deduced from the space group and this value of n. The space group $P6_222$ has 12 general positions. With three molecules per cell, the asymmetric unit of the crystal is one enzyme subunit, and the four subunits of each molecule must be grouped around three mutually perpendicular twofold axes. In other words, the aldolase molecule has 222 (D_2) symmetry, at least to the resolution of our present set of x-ray photographs (about 4 Å). This conclusion verifies the deduction of Eagles

Table 1. Crystal forms of aldolase. The densities for forms I and III in this report are for crystals taken from a single dish. The density of a single crystal modification varies as much as 5 percent depending upon the density of the liquid of crystallization from which the crystal is taken. Thus, the density of form I varies from 1.205 g/ml to 1.255 g/ml as the density of the mother liquor changes from 1.150 to 1.240 g/ml, because of changes in the concentration of ammonium sulfate. However, for crystals taken from the same dish, form I is always more dense than form III by ~0.3 percent. Our results on the change in crystal density with salt concentration in the mother liquor are similar to those obtained by Perutz (*12*) for crystals of hemoglobin. They also explain the discrepancy in the values for the density of form I that appear in the table. V_M is the ratio of the volume of the crystallographic asymmetric unit to the molecular weight of protein within it (*7*).

Form	Space group	Cell dimensions		Density (g/ml)	Cell volume (Å × 10^6)	Molecules per cell (No.)	Molecules per symmetric unit (No.)	V_M (Å^3/dalton)
		a (Å)	*c* (Å)					
I (*3*)	$P6_222$	161*	169	1.25	3.8	6	½	4.0
II (*3*)†	$P2_1$	†	†	1.26	0.78	2	1	2.5
I (*2*)	$P6_222$	161	170	1.19	3.8	6	½	4.0
I‡	$P6_222$	162	170	1.250	3.9	9	¾	2.7
III‡	$P6_222$	96.2	168	< 1.250	1.34	3	¼	2.8

* This value should replace the one cited in (*3*) and (*13*). † Eagles lists the cell dimensions of this form as $a = 164.5$, $b = 57.3$, $c = 85.0$, and $\beta = 102°40'$ (*3*). ‡ This report.

et al. (*3*) from a study of the rotation function on a mercury derivative of form II. The discovery that the four subunits of aldolase are identical to at least 4-Å resolution is interesting in light of evidence that the enzyme contains chemically distinct subunits (*5*, *6*). Lai *et al.* (*6*), for example, have found that an asparagine residue in the COOH-terminal hexapeptide of one chain corresponds to an aspartic acid in the other chain. Apparently neither this nor any other amino acid substitution causes a significant change in the conformations of the subunits.

Comparison of the diffraction pattern of form I (Fig. 1B) to that of form III reveals that the two forms are related. We have confirmed the result of Goryunov *et al.* (*2*) and Eagles *et al.* (*3*) that form I also belongs to the space group $P6_222$ (or $P6_422$). The *c* axial lengths of the two forms are nearly identical, and the *a*-axis of form I is $\sqrt{3}$ times that of the *a*-axis of form III, so that the volume of form I is nearly three times that of form III. Moreover, the *hki*0 sections of the two diffraction patterns are similar: if they are superimposed with their a^*-axes at an angle of 30° with one another, the distribution of spot intensity is seen to be similar (Fig. 1). Since this section of the diffraction pattern may be thought of as the Fourier transform of the *c* projection of the unit cell contents sampled at the reciprocal lattice points this means that the projections of the two cells bear a close relationship to one another. We discuss this relationship below.

In determining the number of molecules per unit cell, *n*, in form I, simple arguments of the type used above for form III are inadequate. This is because the cell volume of form I is greater than that of form III, and consequently $n = 6$, 9, and 12 all yield reasonable values of X_P. Similarly, the parameter V_M (the ratio of the volume of the crystallographic asymmetric unit to the molecular weight of protein contained within it) tabulated by Matthews (*7*) does not restrict *n* to a single possible value, though it casts some doubt on the value of six proposed by Goryunov *et al.* (*2*) and Eagles *et al.* (*3*). Matthews found that V_M ranged from 1.68 to 3.54 $Å^3$ $dalton^{-1}$ for the 116 protein crystals he considered. For $n = 9$ and 12, V_M of form I falls within this range, whereas for $n = 6$ it lies slightly outside the range (Table 1). In view of this, we redetermined *n* for form I by the following two methods.

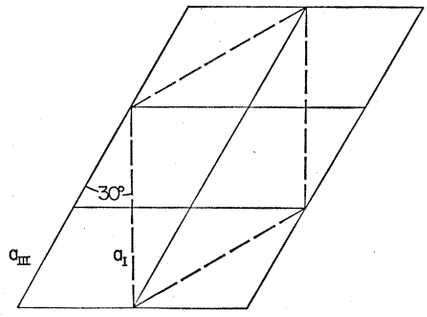

Fig. 2. The *c*-projections of the space lattices for forms I and III. Note that the *a*-axes of the two lattices make an angle of 30° with one another, and that *a* for form I is $\sqrt{3}$ times *a* for form III.

The "method of multiple crystal forms" uses the known value of *n* in one crystal form, along with relative cell volumes and densities, to place limitations on *n* in a second form. In applying the method to form I, we note that form I is more dense than form III, but its volume is not quite three times that of form III (Table 1). Since the protein is more dense than the liquid of crystallization, and since the density of the liquid of crystallization is presumably the same in both forms, the greater density of form I can be achieved only by an increase in the number of molecules per cell by a factor of 3 or more. Thus, *n* must be at least nine in form I. A similar comparison of forms I and II restricts *n* in form I to fewer than 12. Thus, $n = 9$ in form I.

In the "crystal-volume and protein-content method" one determines the mass of protein, *m*, in a large crystal of known volume, *v*. The volume of a large crystal of regular habit is measured under the microscope, as in the method of Low and Richards (*8*). The mass of protein is found by dissolving the crystal in a known amount of water and then by applying the Lowry method (*9*) or by measuring the optical density at 280 or 230 nm (*10*). Then *n* is given by:

$$n = \frac{m N V}{v M} \qquad (2)$$

We applied this method to form I and found $n = 8.5 \pm 1$, based on an average of 13 determinations. In obtaining averages of the results of separate determinations, we applied a weight proportional to the crystal volume because as any dimension becomes less than 0.4 mm the probable error in *v* increases rapidly. It should be noted that this method avoids the assumptions about residual water and salt that are introduced with the usual gravimetric method. The present method can be applied whenever large crystals with regular habits are available.

Our conclusion that $n = 9$ is reinforced by the determination reported by Eagles *et al.* (*3*) of the weight fraction of protein, X_P, in form I crystals. They weighed crystals first when wet and then when air-dried and, after they corrected for the probable mass of remaining water and salt, arrived at a value of 48.5 percent for X_P. When this value is inserted into Eq. 1 along with our observed cell dimensions and a molecular mass of 160,000 daltons, *n* is calculated to be 9.0.

Nine molecules can be packed in a cell of space group $P6_222$ only if three are each positioned at the intersection of three twofold axes and if the six others lie on twofold axes [for example, three on special positions *a* of (*11*) and (*6*) on special positions *i*]. Thus, the form I crystals, like the form III crystals, show that the aldolase molecule has 222 symmetry (although because the diffraction pattern of form I extends less far out into reciprocal space, this information on symmetry applies only at very low resolution).

The similarity, noted above, of the spot intensities of the *hki*0 sections of the diffraction patterns of forms I and III (Fig. 1) can be interpreted in terms of a unit cell for form I that contains three times the number of molecules in a unit cell of form III. Three molecules would be associated with each lattice point of the unit cell of the form III crystal (Fig. 2). Also shown in Fig. 2 is the outline of a cell having all the observed characteristics of the form I crystals: the *a* axial length of this cell is $\sqrt{3}$ times that of the form III cell, and it contains nine molecules rather than three. Moreover, the molecules associated with the lattice points of this larger cell would have the same orientations as molecules in the cell of the form III crystals. Thus, the transforms of the projections would be expected to show similar spot intensities, although the a^*-axes of the two reciprocal cells would be at 30° to one another. This is exactly what is observed in Fig. 1.

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14. Supported by PHS grant GM 16925 and by an Alfred P. Sloan research fellowship to D.E. The early work on crystallization supported by PHS grant GM 11094 to P. Boyer. We thank P. Boyer and R. E. Marsh for helpful discussions. B.H.W. thanks the American Cancer Society for a fellowship. Contribution No. 2731 of the Department of Chemistry, University of California, Los Angeles.

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25 September 1970

Chromatid Breakage: Cytosine Arabinoside–Induced Lesions Inhibited by Ultraviolet Irradiation

Abstract. *Exposure to ultraviolet light can reduce the frequency of chromatid breaks induced by cytosine arabinoside in the DNA synthetic and post-DNA synthetic phases of the cell cycle. This effect can be correlated temporally with a decrease in the uptake of tritiated thymidine after ultraviolet treatment, implying that the genesis of such breakage is intimately related to DNA synthesis and that such synthesis is not confined to the DNA synthetic phase.*

Cytosine arabinoside (ara-C) inhibits DNA synthesis. The drug prevents the reduction of cytidine diphosphate (*1*), interferes with DNA polymerase (*2*), and produces fraudulent macromolecules by incorporation into DNA and RNA polynucleotides (*3*). In cultures of hamster fibroblasts, ara-C produces chromatid breaks in the pre- (G_1) and post-DNA synthetic (G_2) phases of the cell cycle as well as during the DNA synthetic (S) phase (*4*). Deoxycytidine prevents chromatid breakage when added simultaneously with ara-C and decreases the number of breaks when added after a 30-minute pulse of the drug in the S phase but not in the G_1 or G_2 phase (*4*). Since ultraviolet irradiation produces chromatid breakage (*5*), exposure to both ultraviolet and ara-C would be expected to increase the frequency of chromatid aberrations. In contrast to these expectations, exposure to ultraviolet light prior to ara-C treatment markedly decreased the number of chromatid breaks.

Asynchronous cultures of hamster fibroblasts, Don-C (T_C, generation time, 13 hours; G_1 phase, 3.9 hours; S phase, 6.2 hours; G_2 phase, 2.2 hours; and mitosis, 0.7 hour), growing in monolayers (*6*) were treated with ultraviolet irradiation (2537 Å), 3 and 9 erg/mm^2, after removal of media. Immediately thereafter, except as otherwise indicated, replicate cultures were exposed to ara-C (10 μg/ml) for 30 minutes 2.5 hours (G_2) and 4.5 hours (S) prior to harvest. After drug exposure, the cultures were washed three times with Hanks solution, fresh media not containing the drug were added, and incubation was resumed. The cultures were treated with colcemid (0.06 μg/ml) 1 hour prior to harvest to collect metaphases. Sodium citrate (0.95 percent) was added for 30 minutes, the cells were removed with a rubber policeman, and chromosome preparations were made as previously described (*4*). The cells were stained with aceto-orcein. Fifty metaphase cells were counted to estimate chromatid breakage. In this study, chromatid gaps with separations at least the width of a chromatid were considered "breaks." Only lesions which completely dissected the chromatids were counted.

Table 1. Effect of ultraviolet light on cells treated with ara-C (10 μg/ml, 30 minutes).

Ara-C (μg/ml)	Ultraviolet (erg/mm^2)	Percentage of metaphases with N breaks per cell			
		0	1 to 4	4 to 9	10+
		G_2 phase			
None	None	94	6	0	0
10	None	44	50	4	2
10	3	62	38	0	0
10	9	74	24	2	0
None	9	92	8	0	0
		S phase			
None	None	96	4	0	0
10	None	56	40	0	4
10	3	76	22	2	0
10	9	84	16	0	0
10	9*	60	34	0	6
None	9	98	2	0	0

* Ultraviolet irradiation after 30 minutes' exposure to ara-C. All other exposures were just prior to ara-C treatment.

The results of these experiments are shown in Table 1. Treatment with ara-C in the G_2 phase (2.5 hours) caused chromatid breaks in 56 percent of the metaphases. The frequency of breakage was decreased to 38 and 26 percent when the cells were exposed to 3 and 9 erg/mm^2, respectively, prior to ara-C treatment. Ultraviolet irradiation (9 erg/mm^2) did not by itself produce an increase in chromatid breaks. Treatment with ara-C in the late S phase (4.5 hours) produced breakage in 44 percent of metaphases. The frequency of metaphases containing breaks was reduced to 24 percent by ultraviolet irradiation with 3 erg/mm^2 and to 16 percent with 9 erg/mm^2 ($P < .05$). If the ultraviolet irradiation was added after the 30-minute ara-C exposure in the S phase, the number of chromatid lesions was the same as seen after a 30-minute ara-C exposure without ultraviolet irradiation, indicating that ultraviolet light must precede ara-C to interfere with chromatid breaks.

The effect of ultraviolet light was also studied after treatment of the cells in the G_2 and late S phases with 10 μg of drug per milliliter for 1.5 hours (Table 2). In the absence of ultraviolet irradiation, 74 percent of the metaphases had chromatid breaks in the G_2 and 70 percent in the S phase. When the cells