ratus can be made small and compact for mounting on individual flasks in an incubator or on a shaker for adding acid, alkali, or other reagents during incubation of bacterial cultures. Only light leads are needed to service the units and, if several units are to be used, as for replicate flasks, addition will be identical in all flasks if the electrodes are wired in series. (vii) There are no moving parts which need lubrication.

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Reference

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Radiation-Induced Reactions of Potassium Bromide with Air

Pressed discs of potassium bromide (*1*) showed selective absorption in the 4000 to 650-K (*2*) infrared region after irradiation with 1.5-Mev electrons in the presence of air, oxygen, nitrogen, helium, or carbon dioxide. The samples received about 10^{21} ev/g at a dose-rate of about 10^{19} ev/min. In all experiments the potassium bromide "windows" became less transparent as devitrification, with consequent scattering, proceeded. In addition, there was decreased absorbance of the only bands initially present—those centered at 3430 and 1630 K, which were due to adsorbed water or to occluded water retained by the potassium bromide despite careful desiccation, or to both. These results were produced also by heating the discs several hundred degrees. The rate of devitrification decreased as the rate of heating was decreased. The "windows" were restored to the glass-clear condition by repressing. While loss of water and devitrification were the only effects noted for experiments conducted in the three gases last named, in oxygen and in air, radiation-induced chemical reactions occurred.

When a potassium bromide "window" was irradiated in ordinary laboratory air, new bands appeared in the infrared absorption spectrum, with maxima at 1360, 1260, 830, 1440, 1765, and 2340 K, in order of decreasing magnitude. Part of the pattern was unstable and shifted rapidly: peaks at 1385 and 825 K appeared and grew at the expense of the original 1360 and 830 maxima. The rate of shift was greater in humid air. Since the new peaks were characteristic of potassium nitrate dispersed in potassium bromide and in Nujol (*3*), it was interesting to speculate on the precursor. The immediate possibilities were: (i) that scattered "isolated" nitrate ions on the surface of the disc migrated to form potassium nitrate crystals; (ii) that a metastable crystalline phase of potassium nitrate was initially formed on the surface under the influence of the crystalline surface forces of the potassium bromide lattice and subsequently recrystallized. The latter possibility appeared to be the more likely since it was found (*4*) that isotropic, triangular microcrystals of high melting point, which spontaneously changed to the normal anisotropic form of potassium nitrate, were produced on the surface of potassium bromide crystals irradiated in air with polonium alpha rays.

Although nitrogen dioxide alone (the other nitrogen oxides were inert) reacted with potassium bromide in a manner similar to that of irradiated air, the 100-fold greater rate of reaction at room temperature (20-fold greater at 70°C) in the latter case indicated that energy-rich surface dislocations or excited gaseous intermediates were involved in the reaction. At constant dose-rate, it was found that the production of infrared-absorbing species decreased as the contact time of the irradiated air with the salt diminished. Coupled with the fact that discs irradiated in helium at room temperature did not undergo appreciable postirradiation reaction with air, the view that excited gaseous intermediates were involved gained plausibility.

Of the absorption bands listed above, those centered at 1385, 825, and 1765 K could be identified as belonging to potassium nitrate; that centered at 2340 K, which appeared only in windows prepared from chemically pure potassium bromide and not in windows made from Harshaw optical grade potassium bromide, was undoubtedly produced by carbon dioxide arising from the radiolysis of organic impurities; that centered at 1260 K, on the basis of other work (*5*) could be tentatively identified as belonging to potassium nitrite produced by radiolysis of potassium nitrate. Although potassium carbonate has been reported (*3*) to have a very strong absorption band at 1450 K, the 1440 peak here noted did not appear when potassium bromide was irradiated in an atmosphere of carbon dioxide; it did appear when the irradiations were conducted in atmospheres of oxygen or air. Possibly KNO, which has been reported (*6*) to absorb at 1445 K, or KOBr was the absorbing species.

The irradiation of a disc in an atmosphere of dry oxygen produced, in addition to the aforementioned peak at 1440 K, an infrared absorption band with a maximum at 790 K, which was found to be the principal absorption region of potassium bromate when it was dispersed in potassium bromide or Nujol (*3*). This absorption did not appear when the irradiations were conducted in dry or moist air.

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30 September 1957

Mechanism of Immune Hemolysis: Recognition of Two Steps in the Conversion of $EAC'_{1,4,2}$ to E*

In recent publications from this laboratory (*1*) it has been established that the lysis of sheep erythrocytes (E) by Forssman antibody (A) and guinea pig complement (C′) is the result of a series of successive reaction steps, as follows:

$$E + A \longrightarrow EA \quad (1)$$

$$EA + C'_{1,4} \xrightarrow{Ca^{++}} EAC'_{1,4} \quad (2)$$

$$EAC'_{1,4} + C'_2 \xrightarrow{Mg^{++}} EAC'_{1,4,2} \quad (3)$$

$$EAC'_{1,4,2} + C'_3 \longrightarrow E^* \quad (4)$$

$$\hookrightarrow \text{inactive product} \quad (4a)$$

$$E^* \longrightarrow \text{ghost} + \text{hemoglobin} \quad (5)$$

In this scheme C'_1, C'_2, C'_3, and C'_4 represent the four recognized components of complement (*2*). Ca^{++} and Mg^{++} have been found to function in steps 2 and 3, respectively. E* refers to an activated or damaged cell which eventually hemolyzes without further interaction with complement from the fluid phase. The conversion of $EAC'_{1,4,2}$ to E* by C'_3 has been found to proceed without requirement of a divalent cation such as Ca^{++} or Mg^{++}, and hence can take place in the presence of 0.01*M* ethylenediamine tetraacetate (EDTA). As a source of C'_3, either C'_y (guinea pig serum lacking C'_1 and C'_4) or whole guinea pig serum in the presence of 0.01*M* EDTA has been used. The identification of C'_3 as the component which interacts with $EAC'_{1,4,2}$ to form E* (*3*) is based on the observation that $EAC'_{1,4,2}$ can be hemolyzed by R_1, R_2, or R_4, but not by R_3 (*4*).

With the aid of a kinetic theory of the mechanism of steps 4 and 4a, it has been shown that both the rate and extent of reaction 4 are dependent on the square of C'_3 concentration (*5*). Since no lag was detected in kinetic analyses of step 4, it was postulated that this process involves a single reaction, that is, a one-hit mechanism. In order to reconcile this with the dependence of rate and extent of reaction on the square of the C'_3 concentration, it was assumed that the process involves two simultaneous hits by C'_3 (*1*) or that the active form of C'_3 is a dimer in equilibrium with an inactive monomer. However, these assumptions have now become unnecessary, for recent experiments have furnished two independent lines of evidence which indicate that the conversion of $EAC'_{1,4,2}$ to E* requires more than one step.

Before this new evidence is presented, it should be pointed out that the observation that $EAC'_{1,4,2}$ can be hemolyzed by R_1, R_2, or R_4, but not by R_3 (*3*), does not necessarily imply that C'_3 is the only component required to transform $EAC'_{1,4,2}$ to E*. The possibility must be considered that the conversion of $EAC'_{1,4,2}$ to E* requires C'_3, as well as another, as yet unrecognized, component of the C′ system. In our earlier work this possibility was dismissed because the process was believed to be a one-hit reaction.

In recent kinetic studies of reactions 4 and 4a, it was discovered that no lag was apparent in the earlier experiments (*1*) because the technique employed to stop the reaction in the samples at the desired times was not adequate. This difficulty was overcome (i) by working in a cold room at about 5°C instead of room temperature, (ii) by diluting the samples 1/11 instead of 1/3 with ice-cold buffer, (iii) by centrifuging immediately in prechilled (5°C) table centrifuges rather than at room temperature, (iv) by pouring off the supernatant fluids immediately after centrifugation, and (v) by resuspending the cells in fresh buffer before further incubation at 37°C, instead of incubating the sedimented cells in the presence of the original supernatant fluid. The use of a 1/11 dilution of the samples was arrived at after several lower dilutions were tested under conditions i, iii, iv, and v, as shown in Fig. 1. This dilution represents the highest dilution feasible under present experimental conditions.

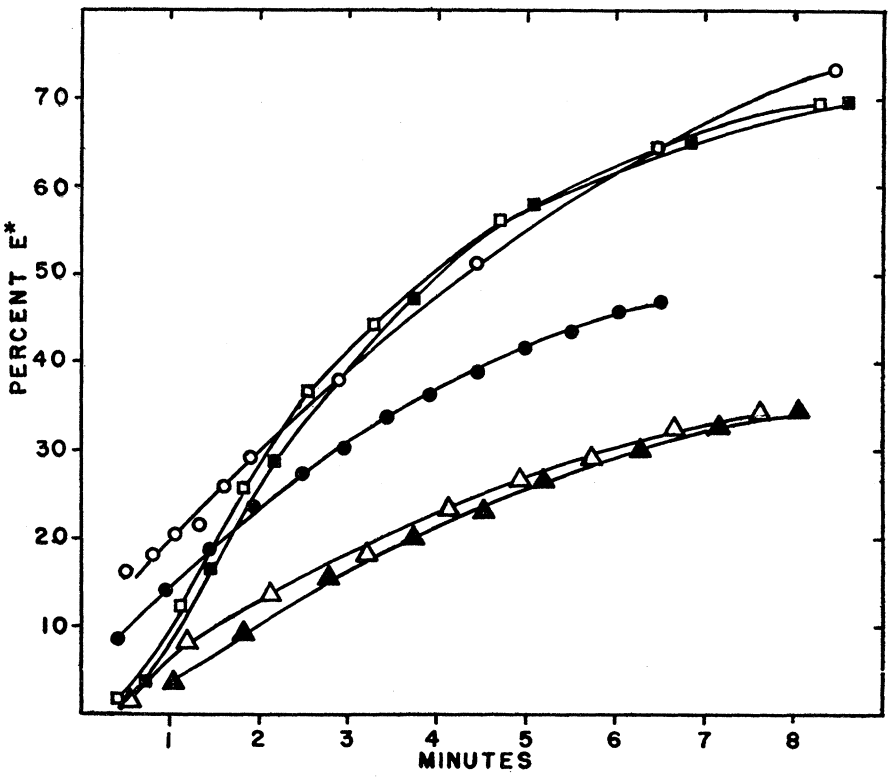

Fig. 1. Kinetics of E* formation from $EAC'_{1,4,2}$. In the descriptions that follow, the term *new sampling method* refers to conditions i, iii, iv, and v described in the text. Open circles: old sampling method, C'_3 1/100 (*10*), experiment No. 082354. Solid circles: old sampling method, C'_3 1/115 (*10*), experiment No. 090154. Open squares: new sampling method, 1.0 ml of sample + 5.0 ml of stopping diluent, C'_3 1/60 (*10*), experiment No. 122156. Solid squares: new sampling method, 1.0 ml of sample + 10.0 ml of stopping diluent, C'_3 1/60 (*10*), experiment No. 122156. Open triangles: new sampling method, 1.5 ml of sample + 3.0 ml of stopping diluent, C'_3 1/90 (*10*), experiment No. 111956. Solid triangles: new sampling method, 1.0 ml of sample + 5.0 ml of stopping diluent, C'_3 1/90 (*10*), experiment No. 111956.

While this new technique may not be perfect, in the sense of effecting complete blockage of further C'_3 action during sampling, it is clear that results obtained in this fashion reflect more closely the actual course of events than do those obtained with the earlier method. With the improved sampling method it was demonstrated that the kinetic curve of E* formation is sigmoidal and not concave to the abscissa as previously claimed (Fig. 1).

This finding indicates that the conversion of $EAC'_{1,4,2}$ to E* involves at least two reaction steps. Hence, an attempt was made to learn, by fractionation of guinea pig serum, whether the components required in these reactions are distinct (*6*). A method was found which led to a separation of two fractions, both of which appear to be necessary for the conversion of $EAC'_{1,4,2}$ to E*. One fraction was obtained by precipitation from whole guinea pig serum on addition of 0.005*M* HCl to *p*H 5.0. After collection by centrifugation, the precipitate was dissolved in 0.04*M* phosphate buffer at a *p*H of 7.1 to a volume equal to the original amount of serum and reprecipitated at *p*H 5.0 with 0.005*M* HCl. The supernatant fluid from the initial precipitation was rendered isotonic and neutral by addition of appropriate amounts of 1.5*M* NaCl and 0.1*M* NaOH. The second fraction was obtained from this solution by addition of methanol to a concentration of 28 percent (by volume). The resulting precipitate was dissolved in a convenient volume (3 to 5 times the original serum volume) of isotonic NaCl and reprecipitated by addition of methanol to 40 percent (by volume). All operations were performed at 0 to 2°C.

The results of five experiments, summarized in Table 1, indicate that the two fractions are far more active in admixture than alone. It appears, therefore, that conversion of $EAC'_{1,4,2}$ to E* requires at least two factors.

However, before this conclusion can be regarded as valid it is necessary to consider the possibility that anticomplementary effects might account for the

Table 1. Reciprocal of the final dilution of reagent required to convert 50 percent of 7.5×10^7 $EAC'_{1,4,2}$ to E* in a reaction volume of 2.5 ml in the presence of 0.01*M* EDTA in 90 minutes at 37°C. In all assays, the cells as well as the C′ fractions were used on the day of preparation.

Expt. No.	*p*H 5.0 ppt.	Methanol ppt.	Mixture	Whole C′
1	< 5	47	260	610
2	36	8	400	720
3	< 5	< 5	260	710
4	95	17	490	720
5	86	18	390	650

results cited in Table 1. Thus, it is conceivable that one of the fractions might contain C'_3 in admixture with an inhibitor, while the other fraction might contain an anti-inhibitor. If this were the case, both fractions would be inactive when used alone, but activity would be restored by admixture. Tests with the fractions described in this paper showed that the acid precipitate, after incubation with whole C′ at 37°C for 30 minutes, strongly inhibited the action of the latter on EA, while the methanol precipitate lacked inhibitory action. These results, however, are not relevant to the system under study—that is, the conversion of $EAC'_{1,4,2}$ to E* by C′ in the presence of EDTA.

An appropriate test would involve incubation of whole C′ in EDTA with the fractions alone and in combination followed by tests of these mixtures with $EAC'_{1,4,2}$ as substrate. This was done with an incubation temperature of 37°C and incubation time of 30 minutes, and with a concentration of the acid precipitate which had been found to be strongly inhibitory to whole C′ in the previous test. No anticomplementary action was observed in the modified test with $EAC'_{1,4,2}$ as substrate, and, indeed, in all cases such treatment enhanced the conversion of $EAC'_{1,4,2}$ to E*.

The results of the fractionation experiments permit two alternative interpretations: (i) Each of the fractions contains a different component of complement; these two components act sequentially to transform $EAC'_{1,4,2}$ to E*. (ii) One of the fractions contains a component of complement (presumably C'_3) which converts $EAC'_{1,4,2}$ to E* by two successive reactions, while the other fraction contains a cofactor or activator for this process.

Further experiments are needed to distinguish between these interpretations. With respect to the first interpretation, since either R_1, R_2, or R_4 is able to convert $EAC'_{1,4,2}$ to E*, it would appear that both of the factors involved in this reaction are distinct from C'_1, C'_2, or C'_4. Furthermore, since R_3 does not convert $EAC'_{1,4,2}$ to E*, at least one of these factors is C'_3. Attempts were made to reconstitute R_3 (at a nonlytic level) by addition of the alcohol and acid precipitable fractions, alone and in combination (in the proportions corresponding to the original guinea pig serum). The three mixtures, as well as whole C′, were assayed for their ability to hemolyze sensitized sheep red cells (EA) in the presence of Ca^{++} and Mg^{++}. The results were expressed as the reciprocal of the final dilution required to yield 50-percent lysis, as judged by visual inspection, and are given as follows: R_3 + methanol precipitate, 120; R_3 + acid precipitate, 350; R_3 + mixture, 750; whole C′, 1500.

This experiment indicates that both the methanol precipitate and the acid precipitate contain C'_3, in the sense that they are lytic with R_3. However, the experiment is inconclusive with respect to the possible duality of C'_3, since the titer of the mixture of the two fractions was only moderately higher than the sum of the titers of the fractions alone.

One of the limitations in the use of R_3 arises from the fact that it does not furnish an excess of C'_1, C'_2, and C'_4 and that the purified fractions may contribute one or more of these components, leading to the formation of $EAC'_{1,4,2}$ of different activity in the different mixtures. By contrast, in the experiments described in Table 1, in which the fractions were tested with preformed $EAC'_{1,4,2}$, further formation of $C'_{1,4,2}$ sites on the cells was blocked by the EDTA.

While it is evident that one of the factors is C'_3, it is not clear whether the other one is a second part of C'_3, an activating factor, or a new component of complement. This question requires further study of the nature of R_3. In addition, it is not known whether the two factors act on $EAC'_{1,4,2}$ in a definite sequence. Finally, it will be necessary to reexamine the properties of $EAC'_{1,4,2}$ with the aim of determining whether a population of cells "in the state $EAC'_{1,4,2}$," when prepared as described by Levine, Mayer, and Rapp (*3*), has progressed, at least in part, beyond this stage by interaction with one of the factors. This possibility must be considered since in the preparation of $EAC'_{1,4,2}$ a small proportion of cells is lysed.

In regard to the nature of R_3, it is pertinent that Da Costa Cruz and De Azevedo Penna (*7*), employing conventional C′ reagents, concluded that C'_3 consists of at least two different substances, on the basis of the differential destruction of C'_3 by formaldehyde on the one hand and by sodium hydrosulfite on the other.

There have been quite a few reports presenting evidence of the existence of complement components other than C'_1, C'_2, C'_3, and C'_4 (*8*). Some workers in the field, including the members of this laboratory, have been reluctant to accept the existence of more than four components because of the limitations inherent in the use of serum fractions which are subjected to destructive treatments and which are recombined in order to reconstitute hemolytic activity. The present evidence on a new component or factor of the complement system is not based on the use of reagents prepared by destructive treatment (*9*).

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4. R_1, R_2, R_3, and R_4 are reagents for the detection of the four complement components and are prepared by selective removal or inactivation of either C'_1, or C'_2, or C'_3, or C'_4, respectively, from whole guinea pig serum.
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9. This work was supported by grants from the National Science Foundation and by contract Nonr 248(17) with the Office of Naval Research.
10. It should be noted that C'_3 concentrations are comparable only within a given experiment, since the reactivity of $EAC'_{1,4,2}$, which varies from experiment to experiment, affects the extent of reaction. The reaction mixtures contained 3×10^7 erythrocytes per milliliter and 5.0 ml of the C'_3 dilutions indicated for each experiment in a total volume of 25.0 ml.

24 September 1957

Banana Feeding and Urinary Excretion of 5-Hydroxyindoleacetic Acid

The diagnosis of malignant carcinoid in man can be made by the presence of markedly increased excretion of 5-hydroxyindoleacetic acid (5-HIAA) in the urine. The increased excretion of this acid in the urine of patients with carcinoid tumors is derived from 5-hydroxytryptamine, which is present in very large concentration in the carcinoid tumors (*1*). 5-Hydroxytryptamine is converted to 5-HIAA by the action of aminooxidases (*2*). The range of excretion values for 5-HIAA obtained from carcinoid patients is 21 to 680 mg/day, as compared with 2 to 9 mg/day for adult normal subjects (*3*).

The 5-hydroxyindoles found in the urine of carcinoid patients and of normal man are derived primarily from the metabolism of tryptophan. The excretion in normal man is relatively constant over wide ranges of tryptophan intake. However, addition of very large amounts of tryptophan to the diet of normal man results in increased excretion of 5-HIAA in the urine. A twofold increase in excretion of this acid has been demonstrated following loading with 3.5 g of tryptophan.

To date, the elevated values of