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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1963

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

OPERATED BY THE UNIVERSITY OF CHICAGO
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USE OF AN ANALOG COMPUTER TO CALCULATE TREATMENT DOSE FOR MULTIPLE FIELDS*

By

L. S. Skaggs and S. D. Savic

Manual calculation of the internal dose in radiation therapy resulting from the application of several beams through different portals is a tedious and time consuming operation. Calculations for different amounts of radiation through the various portals is even more tedious and is seldom attempted. As a result only a few of the possible modes of treatment are investigated, and something less than the optimum is finally adopted.

Many methods have been suggested for automatic or more rapid calculations of internal dose. The important and distinguishing feature of these is generally the means used to represent the dose distribution of a single beam of radiation. Since the calculation is generally done only for a single plane or at least one plane at a time, means are required for the representation of dose with respect to the variables of a plane. Thus dose is a function of two variables, and the variables of depth and width across a plane through the axis of the beam are the ones commonly used.

Automatic calculation, then, requires a function generator of two variables. Many schemes have been proposed for such devices and have been successfully used in technology. The more common include mechanical, optical, and electrical methods, to which should be added digital representations of functions. Mechanical methods generally take the form of a two dimensional cam. Such devices are generally difficult to fabricate and to use, and examples of their use in radiation dose calculation are not known. Optical function generators of two variables generally use a variable density plate. There are however many problems associated with linearity and production of the plates and with measurements of density.

Digital representation of functions is becoming well known. This requires some form of memory and means of operating on the contents of the memory such as is found in the large digital computers. A number of papers have been published and the method has ardent supporters.¹⁻³ Objections, however, have been advanced, based on the granularity of the digital representation, the granularity in beam orientation, and the usual numerical form of output.

While the digital computer is an electrical device, the term electrical function generator is more commonly applied to the analog devices. A very interesting application of one of these was described recently by Howarth and Rich.⁴ They used copper-coated printed circuit boards and developed them in such a manner that the copper was left on the board in a representation of the usual isodose lines of a dose distribution with depth in tissue. The insulating board and copper lines were then uniformly coated with a resistive material to provide some electrical conduction between the copper lines. When voltages proportional to the lines of constant dose

* This paper was presented at the 48th Annual Meeting of The Radiological Society of North America, Chicago, Ill., November 25-30, 1962. Abstract published in the journal Radiology, Volume 80, No. 1, January, 1963.

are separately and simultaneously applied, the resistive material provides an interpolation between the lines. If the function represented is a solution of Laplace's equations, the interpolation is exact. Dose distribution within tissue does not follow such a function, but nevertheless, the interpolation can easily be made accurate within one or two per cent.

In the device developed by Howarth and Rich, each beam of radiation is represented by a different function plate. The function plates are positioned mechanically with respect to an outline of the patient's body, so as to represent the various beams of radiation. Metallic probes on each plate are positioned mechanically with respect to each other, and with respect to an indicating probe on the diagram of the patient's body. Voltages from the various probes are summed in an operational amplifier and show the total dose at the position of the indicating probe. The indicating probe and the attached pickup probes may be moved about to locate points on the same isodose line for the total radiation distribution and the isodose lines plotted. The method is relatively simple and cheap, and gives good results. However, the function generator boards are not easy to make, and large numbers are needed to represent the many possible beams of radiation.

A commercial function generator of two variables has recently become available which uses a similar resistive plate but which is more adaptable to electronic analog methods, since the voltage pickup probe is moved by servo mechanisms in the rectangular coordinate (X,Y) directions.* Since the use of electronic analog rather than purely mechanical methods to move the probes offers much more flexibility in operation, it was decided to investigate its use along with an analog computer in radiation dose calculations.

The function generator is shown in Figure 1. The function plate mounts on a standard X-Y plotter, and the metallic pickup probe is moved by the mechanism that normally moves the pen. The unit on the left is a potentiometer device for conveniently setting the correct voltages on the conductive lines of the function plate. It also contains a nulling meter, and a selector switch which facilitates the comparison and setting of the potentiometer outputs to a set of standard voltages. There are twenty potentiometers and each one is wired to a spring connector that makes contact with one of the conducting lines on the function plate.

The controls on the far right of the plotter unit (Figure 1) include scale factor for both X and Y directions ranging from 0.1 volts per inch to 20 volts per inch; and parallex controls. The latter permit setting the origin of coordinates to any position within the generator space.

The function plates are commercially prepared and consist of a transparent lucite plate about 1/16 inch thick, coated on each side with a uniform conducting material. This coating has a resistance of approximately 20,000 ohms per square. The curves of constant dose are plotted on the plates with a conducting silver ink with each curve brought out to a connecting tab on one edge. Figure 2 shows such a plate with the isodose lines for a 10 x 10 cm field from a 2 MeV Van de Graaff generator. The broad straight line on the left corresponds to 100 per cent and lines are drawn for 95 per cent, 90 per cent, 80 per cent, etc., down to 10 per cent, 7.5 per cent, 6.7 per cent and 3 per cent. The partial line is drawn at 15 per cent to give improved curve following in this region where the lines become farther apart. Because of the density of lines along the edge, only one end is connected and the other is terminated in space. Actually, the plate would

*Electronic Associates, Inc., Long Branch, New Jersey.

Figure 1. Bivariate function generator showing the plotting unit and function plate on the right and potentiometer unit on the left.

Figure 2. Bivariate function generator plate with silver ink isodose lines drawn for a 10 x 10 cm, 2 MeV Van de Graaff field in water at 100 cm F.S.D.

be slightly improved if the termination of some of the lines were made as soon as the free end becomes approximately equidistant from its two neighbors. By alternating the end that is terminated from one edge of the field to the other, the voltage balance is improved, and no effects of finite resistance of the silver ink line are seen. The long low percentage lines are terminated on both ends, where possible, or extended for some distance between connected lines, so they will pick up some voltage from the latter. Connections of the lines on the left are somewhat obscured in the illustration because another function is drawn on the back surface and the connecting lines to the tabs show through the transparent plastic.

The plates are designed to operate at a maximum of ± 100 volts with most functions. Because of the density of lines at the edge of the field in the dose function, it is advisable to limit the voltage to a smaller value to prevent overheating. A value of 10 volts was chosen to represent 100 per cent, with 9.5 volts representing 95 per cent, etc. A supply voltage of 12 volts was provided for the potentiometer unit and this was also used to power a standard voltage divider, providing comparison potentials for the potentiometer.

The analog method of dose calculation is shown schematically in Figure 3. The dose contribution for each field is produced by a separate bivariant function generator, and fed to the control and arithmetic unit. This unit sums the contributions from the various fields and compares

Figure 3. Block diagram of the analog computer.

it with a preselected value. Through the closure of a servo loop, the proper coordinates are fed to each function generator to produce a null between the sum of the doses and the preset dose. The arithmetic unit also computes the X,Y coordinates of position in patient space where this preselected dose value occurs, and feeds the information to the plotter, so that an isodose curve can be plotted representing the total radiation.

There are many possible advantages to the complete analog approach to treatment calculation and planning. These include:

1. Servo-loop control so that the isodose lines for the total radiation field are plotted directly within an outline of patient space.
2. Generation of any desired radiation field size from only a few function plates.
3. Generation of oblique fields for any angle from a normal field function plate.
4. Generation of fields produced by wedge filters.
5. Correction for different focal skin distances.
6. Orientation of radiation fields within patient space by electronic means.

Most of these can be tested with a single function generator and a simplified computer circuit. This was the approach selected: the computer was set up to reproduce a single field of radiation, and the accuracy and flexibility of this method studied.

It is first necessary to select a coordinate system and a means of closing the servo loop for reproduction of an isodose curve. As explained previously, a function generator of two variables is necessary, but during the reproduction of a single isodose line, the value of dose remains a constant. Then X becomes a function of Y or vice-versa as illustrated in Figure 4 where the curve for 40 per cent dose is shown being traced. The variables X and Y are not convenient

Figure 4. The polar coordinate system used to reproduce the isodose curves.

for computer use, because no matter which is chosen as the independent variable, the dependent variables will be multivalued and not well determined in some regions. This can be seen by examination of Figure 4. A better choice would be the polar coordinates, θ , and its dependent variable r , with the origin at the surface on the axis of the field. This location of the origin is not strictly necessary for any given curve as may be seen by reference to the figure, but it does work out best for a family of curves. Now if a reference voltage is chosen to define the curve to be traced (4 volts in the case of Figure 4), and if a servo loop is closed to drive r to increasing

values until a balancing voltage is picked up by the function generator probe, then a method is available for automatically forcing the probe to follow the isodose line. It is only necessary to vary θ manually over the range of $+90^\circ$ to -90° and r will be forced to follow the value of the curve. Of course, the function generator and the X-Y plotter operate in cartesian coordinates, but it is easy to generate $r \cos \theta = X$ and $r \sin \theta = Y$ by analog methods. Then X and Y are fed simultaneously to plotter and function generator. With both set to the same scale factors, the probe follows the desired curve and the pen of the plotter draws a faithful record of the movement of the probe.

The simple analog computer circuit diagram for reproduction of single field isodose patterns is shown in Figure 5. There are several good textbooks on analog computation but a few words here on terminology may be in order.⁵⁻⁷ All voltages are measured and stated with respect to ground. Only the high side of a circuit is shown, the return at ground potential is taken

Figure 5. Circuit diagram of the analog computer used to reproduce single fields of radiation.

for granted. The triangles represent operational amplifiers—electronic amplifiers with a D.C. gain of 10^4 to 10^8 , with the output of opposite sign to the input and with a response decreasing

with frequency so that unity feedback can be used. The small circles indicate potentiometers which are connected across the incoming line from high side to ground. A variable sliding contact picks off any fraction of the incoming voltage between zero and unity and this becomes the output voltage. Resistors and capacitors are represented in the normal manner; other elements of the circuit are labeled.

An operational amplifier with an input resistor of 1 M (megohm) and a feedback resistor of 1 M has a net gain of unity and only changes the sign of the signal; it is known as an inverter. If the input resistor is 0.5 M and the feedback resistor 1 M, the gain is 2. Frequently, as will be seen in a later diagram, the resistors are not shown and a small numeral beside the input indicates the net gain. An operational amplifier with 1 M feedback and two inputs, each with 1 M resistors, adds the two inputs directly and is known as a summer. The amplifier on the far right in the dotted box is operating in this mode. An operational amplifier with a capacitor in the feedback loop acts as an integrator—the output is the negative time integral of the input voltage. Two such units are seen on the left; the lower one is acting also as a summer (because of the two inputs, and not because of the connection through the 50 K resistor).

The resolver is an analog computer element used to produce the sine and cosine of an angle, each multiplied by another variable. It is a potentiometer-like device with specially shaped and wound resistor cards to reproduce the trigonometric functions. Two contact arms spaced 90° apart bear on the resistance cards, and are rotatable through a full 360° . One arm produces the sine function and the other the cosine function of the angle represented by the position of their common shaft. When the resistance cards are supplied the positive and negative value of a voltage such as r and $-r$, the outputs are $r \sin \theta$ and $r \cos \theta$. These, as mentioned before, are equal to the cartesian coordinates Y and X , and are just the functions needed to drive the plotter and the bivariant function generator. For the moment, the components in the dotted box should be ignored.

The two amplifiers below the function generator act as a current unloading circuit for the function plate, and it is very important that they be used. The variable feedback resistor across the two units should have a value of about 1 M and consist of a variable resistance of 50 K in series with a fixed resistance of about 980 K. The output of the second amplifier is just twice the input of the first. Since the input junction of the first amplifier is maintained within a fraction of a millivolt of ground potential, all the current flowing through the input 1 M resistor is supplied through the variable resistor if the latter is properly adjusted. This can be determined by lifting the probe from the function plate and observing a voltmeter connected to the output of the first amplifier. With proper adjustment the voltage will neither rise nor fall, due to the 0.01 mfd. capacitor connected from probe to ground, but will remain at the same value for a minute or so. If, however, the probe is moved to another position and touched to the function plate, the meter will immediately take up the new position. This unloading circuit also prevents the generation of noise due to vibration of the probe as it is moved over the surface of the function plate.

The power supply (12 volts) is the common source for the function generator, the voltage divider used in setting up the potentials on the isodose lines, and the reference voltage controlled through the potentiometer. Use of a common source for all three eliminates the uncertainty of unstable voltages.

It will be noted that the negative voltage proportional to per cent dose divided by 10 is summed with the positive reference voltage in the first integrator. The output is then fed into a

second integrator. The use of a single integrator in a servo feedback loop has been reported previously,⁸ but the use of two integrators is unique so far as we know. The use of two integrators in series, with the output of the second connected back to the first through an inverter and with a resistive feedback across the first will be recognized by computer experts as a damped linear harmonic oscillator. Net gain, and therefore the period of the oscillator, is controlled by the potentiometer in the output of the second integrator. The damping factor is controlled by the potentiometer in the feedback path around the first integrator. With proper adjustment of the two potentiometers, the system is critically damped, and rapidly seeks the proper null point without overshoot or oscillation. Since the second amplifier operates as a pure integrator, the D.C. gain is infinite and there is no residual error. Since the gain varies through the function generator—being high where the isodose lines are close together—it is necessary that the gain around the servo loop be adjusted as the curve is drawn. This is no problem, however, and the curves are rapidly traced. In closing the servo loop, attention must be paid to the sign of the voltages. It is necessary that a voltage picked up by the probe with an absolute value greater than the absolute value of the reference voltage result in an increasing value of r . The necessity of this condition can be seen by referring to Figure 4.

One important test of an analog dose calculator is its accuracy, and its ability to interpolate between plotted values of dose. This was checked by using the simple computer to reproduce the isodose lines of a single field and to draw lines at smaller intervals. Figure 6 shows such a re-

Figure 6. Reproduction and interpolation of 10 x 10 cm field from a 10 x 10 cm function plate. 2 Mev Van de Graaff, 100 cm FSD.

production of a 10 x 10 cm field reproduced from a function plate for a 10 x 10 cm field similar to that of Figure 2. The dotted line added to the tracing at 20 shows the correct position of the 20 per cent line. The position as drawn by the computer is in error by less than 1 per cent. At other levels of dose the deviation was too small for a line to be drawn. The lines between 20 per cent and 30 per cent dose are interpolations at 1 per cent intervals between the 20 and 30 per cent lines of the function plate. It is obvious from the plot that the interpolation is essentially correct.

The above tracing was made with scale sensitivity of the plotter and function generator set to the same value. Of course, if the Y scale factor in the plotter were set at half the Y factor of the function generator, the plotted curve would have only half the width of the exact reproduction. This would nearly represent the dose distribution for a 5 x 5 cm field. With a slight further adjustment of the Y or width of field scale factor, and an adjustment of the X or depth of field factor, the representation can be made very good.

Figure 7 shows an 8 x 8 and an 8 x 10 cm field, both plotted from a 10 x 10 cm function plate (the bar under the dimension indicates the width of the rectangular field in the plane of the figure; the other dimension is normal to the figure). Again the dotted lines show the correct positions of

Figure 7. An 8 x 8 cm field on the left and an 8 x 10 cm field on the right both reproduced from a 10 x 10 cm function plate. 2 Mev Van de Graaff, 100 cm FSD.

the isodose lines as determined by actual measurement in a phantom. The agreement is quite satisfactory. Figure 8 shows 5×5 and 15×15 cm fields generated by a 10×10 cm function plate. The accuracy is not quite as good as before but this is a larger factor than should normally be

Figure 8. A 5×5 cm field and a 15×15 cm field both reproduced from a 10×10 cm function plate. 2 Mev Van de Graaff, 100 cm FSD.

used. Figure 9 shows 5×5 cm and 6×6 cm fields generated by a 5×5 cm function plate. These are much more satisfactory, but generation of an 8×8 cm field from a 5×5 cm plate as illustrated in Figure 10 is again too large an expansion. This is rather easily seen at the edges of the field near the surface. Function plates are easily prepared for 5×5 , 10×10 , and 15×15 cm fields, and these are sufficient to generate any sized rectangular field in common use for multiple field therapy.

Referring back to Figure 5, two potentiometers will be seen immediately within the dotted box. These are used to adjust the width and depth of field ratios. The function generator was set at a sensitivity of 0.2 volts per inch in both X and Y; this was 2.5 times the sensitivity of the plotter. The potentiometers then have a range of at least two in field size in either direction. The potentiometers can be calibrated with the aid of a few measured fields, and thereafter any sized field may be produced. Figure 11 shows the width of field attenuator settings determined for a 10×10 cm function plate. The abscissa is the attenuator setting and the ordinate is the width of field normal to the plane of the plot. The various families of curves are for the appro-

priate width of field in the plane plotted. The points on the curves show settings determined from measured isodose distributions. Figure 12 shows the same type of curves for depth of field attenuator settings. In this case, there is no distinction between width in the plane, and normal to the plane of the plot.

Figure 9. A 5 x 5 cm and a 6 x 6 cm field both reproduced from a 5 x 5 cm function plate. 2 Mev Van de Graaff, 100 cm FSD.

It is also rather obvious that adding a fraction of the Y variable to the X variable in the plotter as a set of curves is being drawn, will result in a skewed effect in the isodose lines. This is suggestive of the effect of oblique incidence of a beam of radiation on a surface. Referring back to Figure 5, the additional circuitry shown in the dotted box was installed in the computer to check the possibility of generating curves for oblique incidence. As in the previous case of generating variable field size, the modifications to the circuit are made in that part supplying the function generators, since, when several function generators are used for multiple fields, it must be possible to adjust each one independently. It will be seen from the circuit diagram that an inverter has been added in the Y_1 line to yield both + and - values of Y_1 . A double throw switch allows either sign of Y_1 to be chosen depending on whether the oblique angle of incidence is positive or negative. A minor improvement in circuitry could be provided here, which would connect a grounded 1 M resistor to the input side of the inverter when the switch shown is not thrown in that direction. In this way, the load on the potentiometer in the Y line is

not changed.

The positive or negative value of Y_1 is fed through an inverter and then through a potentiometer. The latter determines the value of the oblique angle, the angle increasing nearly linearly with potentiometer setting up to a value of about 45° . The correct setting of the potentiometer is determined empirically by comparison with a few measured isodose curves for oblique angles from which a curve can be plotted of angle versus potentiometer setting. The final amplifier on the right (in the X_1 line) acts as a summer to add the contributions from the X and Y directions.

Figure 10. An 8×8 cm field reproduced from a 5×5 cm function plate. 2 Mev Van de Graaff, 100 cm FSD.

Figures 13 and 14 show the generation of 8×8 cm fields for oblique angles of incidence of 20° and 45° . Both curves are generated from a 10×10 cm function plate. Again the dotted curves showing the correct position of the isodose lines indicate that the accuracy of reproduction is quite satisfactory.

The skewed isodose curves of oblique incidence are suggestive of the type of curves obtained when a wedge filter is placed in the field above the phantom. Accordingly, the reproduction of curves for wedge filters using the techniques described above was tried. While the results were generally satisfactory in the depth, they departed from the observed values by a considerable amount in the region near the surface. More accurate results can probably be obtained using a diode function generator (of one variable), and a multiplier. Since the simple computer available

did not include arithmetic units of this type, and since a large general purpose computer will soon be available, further work on this aspect of dose calculation was deferred.

It should also be possible to generate curves for different focal skin distances using a function plate prepared for a given distance. Empirically determined factors for different tissue depths can be set up in a diode function generator. These can then be read out by supplying the diode function generator with the bivariate function generator depth variable. The resulting correction factor may then be multiplied into the output of the bivariate function generator to obtain the corrected dose.

Figure 11. Width of field attenuator settings for the 10 x 10 cm function plate.

Most of the development of a dose computer is completed when a satisfactory method of reproduction of any required size or shape of field is available. Figure 15 shows in outline form the requirements for a complete system. As stated previously a separate bivariate function generator must be provided for each field to be used in the summation. Each function generator must have its own field shaping and unloading circuits. In addition, each function generator must be provided with a circuit for rotation of its rectilinear coordinates with respect to those of the plotter. This allows each field of radiation to be positioned at any angle with respect to the axes of the patient outline. Summation of the dose contributions from each field is done in the first integrator. The servo loop is closed through two integrators and the rectilinear coordinates are formed by a resolver, as was done in the reproduction of single fields.

Rotation of the axes of a rectilinear coordinate system is done rather easily by analog computer techniques. Advantage is taken of the well known relations

$$X_1 = X \cos \theta_1 + Y \sin \theta_1$$

$$Y_1 = Y \cos \theta_1 - X \sin \theta_1$$

where

X, Y = coordinates of a point in the fixed system

X_1, Y_1 = coordinates of a point in the rotated system

θ_1 = angle of rotation of X_1, Y_1 axes with respect to the corresponding X, Y axes.

Operation of the analog circuits may be followed by reference to Figure 16 and the two resolvers on the far right indicated by R_1 . The dotted line between the two indicates that they are ganged together on the same shaft so that both are set for the same angle, θ_1 in this case. The resolver

Figure 12. Depth of field attenuator settings for the 10 x 10 cm function plate.

section on the right is fed with + and - values of X and produces the functions $X \sin \theta_1$ and $X \cos \theta_1$. In a similar manner the section on the left produces $Y \sin \theta_1$ and $Y \cos \theta_1$. Amplifier 27 sums the functions $X \cos \theta_1$ and $Y \sin \theta_1$ to produce the new variable X_1 . This and the new

variable Y_1 , produced in a similar manner, are then fed through the field shaping circuits before going to the inputs of the function generator.

Operation of the computer as set up to sum two fields may be understood by reference to Figures 16 and 17. The outline of the patient's body with suitable reference landmarks is

Figure 13. An 8 x 8 cm field sloping at 20° reproduced from the 10 x 10 cm function plate.

sketched on the chart of the X-Y plotter. This fixes the directions of the principal axes of the computer (X and Y) with respect to the patient.

The treatment scheme of Figure 17 was chosen to illustrate the computational ability of the computer rather than as an example of an actual case treated. Both normal and oblique incidence are illustrated and different doses are delivered through the two portals. One isodose distribution is generated by a 5 x 5 cm function plate, the other by a 10 x 10 cm plate. The 5 x 8 cm field is at zero degrees to illustrate the origin and direction of the rotation of coordinate axes of the function generators.

After power has been applied to the computer for 15 minutes, the operational amplifiers are each zeroed so that the output of each with no voltages applied at the input remains at zero. The proper function plate is then chosen for each function generator according to the probable field sizes to be used, and the plates are placed on the plotting surfaces of the generators. The switch at the input to Amplifier 19 is thrown to the 1 megohm position making the amplifier a

summer. The same is done for the switch at the input to Amplifier 14, and Potentiometer 19 is set at zero. Potentiometers 20 and 21 are also set at zero. These adjustments set the plotter pen and the function generator probes at the origin of coordinates. These can be checked by rotating the resolvers, R , R_1 or R_2 ; if there is any motion of pen or probes, potentiometer settings and amplifier balances should be rechecked.

The unloading amplifier circuits, consisting of Amplifiers 17 and 22 (or 13 and 18), are now balanced by the method previously outlined. Now the potentials of the silver ink lines on the function plates may be adjusted. The standard voltage divider is connected to the function generator

Figure 14. An 8×8 cm field sloping at 45° to the skin surface reproduced from the 10×10 cm function plate.

to be adjusted and the switch on inverter 5 thrown in the proper direction. The potentiometers of the function generator are then adjusted by the procedure specified by the manufacturer.

The next step in the procedure is the orientation of the beams of radiation with respect to the patient outline. The point of crossing of the beams is chosen and with all X and Y values still at zero, the pen of the plotter is placed at this point by manipulating the plotter's parallax controls. In reality this operation establishes the center of the polar coordinate system. Then the directions of radiation beams are set up by adjusting the resolvers R_1 and R_2 . In the case of Figure 17, R_1 has the value 0, and R_2 a value of about $+110^\circ$.

The depth of the point of intersection in each beam is estimated and the parallax controls

of each function generator are adjusted to bring the probe to that depth, and on the centerline of the isodose curves of the function plate. Then the field shaping potentiometers are adjusted to produce the desired size of radiation field and the correct angle of incidence.

The adjustment of beam orientation can now be checked. Potentiometer 19 is increased and the resolver R rotated to position the pen of the plotter on the skin at the midpoint of one of the radiation fields. The probe of the corresponding function generator should then be at the surface

Figure 15. Functional diagram of analog computer to sum a number of fields.

of the isodose plot and on the centerline of the field. If it is not, the X parallax control of the function generator is adjusted to bring the probe to the surface, and the corresponding resolver rotated to bring the probe to the center of the field. As Potentiometer 19 is reduced to zero, the probe should move along the centerline of the isodose plot, and the pen should move along the

axis of the beam in patient space. If this does not occur, the initial adjustments should be re-checked, particularly the adjustment of Y parallax of the function generator to locate the probe along the central axis of the field when the pen is at the origin of polar coordinates. Following this operation, the orientation of the other beam of radiation is checked and corrected.

The relative amounts of radiation to be delivered through each portal are now chosen, and the values put into the computer. It is best to normalize the most intense beam at 100 r, and to

Figure 16. Detailed circuit diagram of an analog computer to sum two fields.

refer other beams to this one. This potentiometer is set at 100 (Potentiometer 14 in the case illustrated). Then for the case illustrated, Potentiometer 13 is set at 60. With Potentiometer 15 set at zero and Amplifier 19 acting as a summer, the position of the hot spot is located. This is done by adjusting Potentiometer 19 and Resolver R until the position giving the highest reading on the voltmeter, V, is obtained. This position is marked on the chart by lowering the pen momentarily. The meter reading in volts is taken and this value, multiplied by ten, is the dose delivered at the hot spot per 100 r through the reference portal. In the case illustrated; it was 108 rads.

The computer can easily be set up to draw the isodose lines in patient space either at arbitrary levels of dose referred to the stated dose through each portal, or in terms of percentage of the maximum dose at the hot spot. In the former case the proper reference voltage (on the scale 1 volt = 10 r) is provided through adjustment of Potentiometers 15 and 16. In the latter case Potentiometer 16 is set at 100 and Potentiometer 15 is adjusted to give a null at the hot spot. This can be checked by throwing the ganged switch in the input of Amplifier 19 to the

Figure 17. Isodose lines drawn by the computer showing the summation of two fields of radiation.

reference voltage, and adjusting for a null on the voltmeter. (It might be advantageous to make the two switches separately acting instead of ganged so that Amplifier 19 is maintained in summing logic for this operation.) Then percentages of the hot spot dose are obtained by adjusting Potentiometer 16 and reading the percentage directly.

The system is now nearly ready to trace the curves. The only difficulty is that the origin of rotational coordinates is at the point of crossing of the two fields, instead of at the hot spot which is the center of symmetry of the isodose curves to be drawn in patient space. The center of rotation is easily shifted by adding a constant X and Y to the coordinates of the center of rotation previously established. This is done with the summing Amplifiers 23 and 24 and Potentiometers 20 and 21. Of course the polar coordinate system must be returned to the origin by setting Potentiometer 19 at zero before this adjustment is made. The adjustment is not critical.

The dose level for the first isodose line is now selected and set into Potentiometer 16. Potentiometer 19 is set at some small value thought to approximate the position of the isodose line. With the switches in Amplifier 19 set to convert it to an integrator and to supply it with the negative reference voltage, the servo loop is now closed by switching Amplifier 14 to act as an integrator. The pen should immediately seek the selected isodose line and come to rest. The line can be traced then by rotating the resolver R through 360° . It is best to make a trial run with the resolver before the pen is lowered on the chart.

Best results in drawing curves are obtained when the pen comes promptly to the desired position with no oscillation or overshoot. The former condition is obtained by adjustment of Potentiometer 17, increasing its value will decrease the period of response of the system. The period should not be too fast, but should be fast enough to ensure that the pen is following the correct path at the rate the trace is driven by rotation of the resolver R. At the same time, Potentiometer 18 should be adjusted so that the motion is critically damped. This is the position where the pen and probe system just fails to oscillate after one of the probes has been forcibly displaced. Since the gain of the system depends on the dose gradient in the function generators, it will, in general, be necessary to vary the gain potentiometer (17) as the curve is traced.

Some other problems may be encountered in tracing the curves. For example, the 70 per cent curve on the left edge of the 8×8 cm field is essentially multiple valued in r over part of its range, and will be very difficult to generate from a center at the hot spot. If instead, the center of rotation is shifted to the region of the 82 per cent line on the axis of the 8×8 cm field, then the r as a function of θ becomes well defined and the curve is easy to plot. The center of rotation is easily shifted using Potentiometers 20 and 21, and this use introduces no problems. Trouble will also be experienced with the 82 per cent and 90 per cent curves near the 8×8 cm portal. Here the gradient of dose is positive with respect to Δr , and the system is unstable. The center of rotation must be shifted to the region of the skin and the axis of the beam to obtain a negative dose gradient.

One of the advantages of this system of calculation is the ease of changing field size and orientation. It is not necessary to plot out the entire pattern of dose distribution for each trial configuration as has been done in Figure 17. Generally a few strategically placed curves will indicate whether the pattern has potential value, and will indicate the direction of the required changes. While trying various configurations, it is not generally necessary to start each with a new chart. When a variable is changed, however, it is necessary to go back to that point in the procedure and follow it through to the end. For example, if the field size is changed, it is necessary that the probe and surface of the isodose plot on the function plate be relocated on the skin, and the hot spot and its dose value redetermined; but it is not necessary to redetermine the point of intersection of the beams or the constants of the other beams.

Figure 18 shows the system as set up with two function generators and a plotter. The operational amplifiers are Philbrick* USA-3 and K2-X. Generally the USA-3 is more satisfactory but it is also more expensive. The K2-X can be used where the current requirements are 3 milliamperes or less, with some sacrifice in stability. They are not suitable for driving a large load. The USA-3's are conveniently packaged as groups of ten known as Model K7-A10 manifold. One

* George A. Philbrick Researchers, Inc., 127 Clarendon Street, Boston 16, Mass.

of these was used, powered by a Philbrick R-300 stabilized power supply.

There are 26 amplifiers in the circuit of Figure 16. A few could be omitted e.g., amplifiers 5, 2 and possibly 1. Oblique incidence circuits would probably not be required on all function generators. In this way, 30 amplifiers would be sufficient for a computer providing three field capability. The potentiometers are ten-turn, 20,000 ohm wire-wound units. The resolvers are also wire-wound and are 20,000 ohms per quadrant or 10,000 ohms positive or negative terminal to ground.

Figure 18. The analog computer as set up in the Argonne Cancer Research Hospital. The plotter is on the left and the two function generators on the right.

Summary

Use of an analog computer to summarize the dose distribution of two intersecting beams of radiation has been demonstrated and the method indicated for its extension to three or more beams. These may all intersect at a single point or not. A high degree of flexibility in simulating various types of radiation beams has been demonstrated and possibilities indicated for its extension with a more sophisticated analog computer. Calculations for rotational therapy should also be possible with the latter computer. These will be investigated and reported on at a later date.

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PREPARATION OF COBALT-60 OF VERY HIGH SPECIFIC ACTIVITY,
300 CURIES PER GRAM, FOR TELETHERAPY*

By

L. H. Lanzl

An ever-present problem in therapy with external sources of radiation is that of minimizing the penumbra of the beam. The dose fall-off at the edge of a radiation beam is due to the geometrical penumbra and to scattering within the irradiated material. Two factors which influence the width of the penumbra are the design of the collimator and the size and shape of the source. This paper deals only with the source factor.

In general, the smaller the source area which faces the patient, the smaller will be the penumbra of the radiation beam incident on the patient. However, to be useful for therapy, the activity of the source must also be high enough to produce an adequate dose rate at the tumor site. These two requirements necessitate the attainment of a high specific activity of the source material.

Some aspects of the preparation of a cobalt-60 source which have made it possible to minimize the area while providing a sufficient output for therapy follow.

Cobalt-60 is produced by neutron activation of cobalt-59 using a reactor as the source of neutrons; i.e., the cobalt-59 nucleus captures a neutron, producing cobalt-60. The cobalt-60, in turn, decays by the emission of a beta particle followed by two gamma rays in cascade. An isomer of cobalt-60 is produced which has a 10.1 minute half-life. (See Figure 1.)

The cobalt-60 produced during the course of activation of cobalt-59 is not immediately removed from the neutron environment, and a loss of cobalt-60 may therefore occur if it, in turn, also captures a neutron, thus forming cobalt-61. The two states subject to this type of loss, or burn-up, are the ground state of cobalt-60 with a capture cross section of six barns, and the 10.1 minute isomer with a cross section of 95 barns.¹

Figure 2 is the mathematical expression used to calculate the specific activity of cobalt, taking into account the diminishing amount of cobalt-59 available with time, the decay and burn-up of cobalt-60.

Figure 3 represents the calculated activation of cobalt-60 in curies per gram versus time in years for four values of the undepressed neutron flux; namely, 5×10^{13} , 10^{14} , 3×10^{14} , and 5×10^{14} neutrons per cm^2 per second. An activation cross section of 38 barns was used² for cobalt-59, with a burn-up cross section of either zero or six barns for cobalt-60. A half-life of 5.27 years was used for cobalt-60. (In comparing the results of the assumption of zero barns and the measured value of six barns for the burn-up cross section, one can see that, for a neutron flux of 10^{14} or less, that value does not make too much difference. However, at a flux of 3×10^{14} or greater, and after a year and one-half, both the value of the maximum specific activity and the time at which the maximum is reached are greatly influenced. Thus, at the higher flux values the burn-up cross section should not be ignored.) These curves do not take into ac-

* This paper was presented at the Tenth International Congress of Radiology in Montreal, Canada, August 20-September 1, 1962.

Figure 1. Decay scheme of cobalt-60.

count the burn-up of the 10.1 minute isomer of cobalt-60, since this state has a negligible product of neutron capture cross section and half-life, compared with its ground state. The rapid rise which occurs at the beginning of the activation curve diminishes as the time increases. With the higher values of flux, a peak is reached in less than five years.

The problem of self-absorption of gamma rays within large sources of simple geometrical

$$N_{60} \cdot \lambda_{60} \left[\frac{\text{curies}}{\text{gram}} \right] = \frac{\sigma_{59} \phi N_{59} \lambda_{60}}{\phi (\sigma_{60} - \sigma_{59}) + \lambda_{60}} \left(e^{-\sigma_{59} \phi t} - e^{-(\lambda_{60} t + \sigma_{60} \phi t)} \right)$$

where N_{59} = initial amount of Cobalt-59 $\left[\frac{\text{atoms}}{\text{gram}} \right]$
 N_{60} = amount of Cobalt-60 $\left[\frac{\text{atoms}}{\text{gram}} \right]$
 σ_{59} ; σ_{60} = activation cross sections for Cobalt-59; Cobalt-60 $[\text{cm}^2]$
 λ_{60} = decay constant of Cobalt-60 $[\text{sec}^{-1}]$
 ϕ = neutron flux $\left[\frac{\text{neutrons}}{\text{cm}^2 \text{ sec}} \right]$
 t = time $[\text{sec}^{-1}]$

Figure 2. Mathematical expression for the calculation of the production of cobalt-60 in a nuclear reactor.

Figure 3. Calculated activation of cobalt-60 for four values of undepressed neutron flux for burn-up cross section of $\sigma_{60} = 0$ and $\sigma_{60} = 6$ barns.

shapes has been discussed by Dixon.³ Using Dixon's absorption coefficient of $\mu = 0.35 \text{ cm}^{-1}$ for cobalt, calculations were made to show the effect of self-absorption of cobalt-60 gamma rays in a source. The upper curve of Figure 4 shows the per cent transmission (left side) versus the thickness of the source (see Ref. 4). The second curve shows the activity (right side) required to obtain an effective 1,000 curie source (e.g., the equivalent of a source having an activity of 1,000 curies without any self-absorption) versus thickness. The lower set of curves gives the diameter of the front face of an effective 1,000-curie source having the shape of a solid right cylinder, versus the thickness of that source. The four curves in this set are plotted for specific activities of 50, 100, 150, and 200 curies per gram. For a given specific activity it is readily seen that, at a diameter of about 2.5 cm, a slight increase in length results in a drastic reduction in diameter which, in turn, produces a smaller penumbra. However, little reduction of diameter is gained by extending the length of a source to more than 3 cm.

On this basis, it was decided that the source at the Argonne Cancer Research Hospital should be a solid right cylinder 3 cm in length by 0.75 cm in diameter. Interpolation from Fig-

Figure 4. Self-absorption of cobalt-60 gamma rays. Upper curve: Per cent transmission versus thickness of source (scale on left). Center curve: Activity required to obtain an effective 1,000 curie source versus thickness (scale on right). Lower curves: Diameter of front face of an effective 1,000 curie source versus thickness of source (scale on left).

Figure 4 indicates that, for a source of this size, the specific activity per effective kilocurie of cobalt-60 is 140 curies per gram.

In the neutron activation of a sizable piece of cobalt, one must allow for neutron self-protection and flux depression of the source. The combination of these two effects on a single source such as ours resulted in an effective flux equal to 45 per cent of the undepressed neutron flux.⁵

To increase the induced activity, these effects can be reduced somewhat by using a divided source; namely, a number of smaller pieces, irradiated separately from each other, and then stacked to form the same overall geometrical shape as that of the single source. For example, the activity produced in fifteen separate disks,⁶ each 2.0 mm by 0.75 cm, relative to a single solid piece of cobalt was found to be 1.55. The geometry used during activation which produced

this diminution of self-protection and flux depression consisted in placing the cobalt pieces on the periphery of a cylinder. This geometry (Ref. 6), with slight modification, is the one which we employ for the production of kilocurie sources.

The holder used in activation of kilocurie sources contains a hollow inner cylinder which allows water coolant to pass over both inner and outer surfaces of the holder since, during activation, the cobalt becomes heated due to absorption of reactor gamma rays and to the capture gamma rays of cobalt. For each neutron absorbed, 7.7 Mev of capture gammas are produced in the cobalt. Fortunately, not all these rays are absorbed in the cobalt itself, much of the energy being expended elsewhere in the reactor.

When the 45 per cent effective flux is multiplied by the ratio of 1.55 obtained by using the divided source, the effective flux for the divided source is found to be raised to 70 per cent, which makes possible the attainment of the high specific activity aimed for in therapeutic application. Thus, for example, an undepressed flux of 1.43×10^{14} neutrons per cm^2 per second would result in an effective flux of 1×10^{14} .

The activation of such a divided source has been carried out for us at the Materials Testing Reactor in Idaho, U.S.A. Under actual reactor operations it is not usual to monitor the flux at the position of the source on a continuing basis. The flux at any particular time and point will depend on the power level of the reactor, the distribution of fuel and control rods, and added neutron-absorbing materials within the reactor, especially if they are close to the subject point. Figure 5 is a photograph of the disks taken through a periscope at the Argonne National Labora-

Figure 5. Photograph of activated cobalt disks. Corrosion caused by penetration of small amount of cooling water into holder. Original size of disks 0.75 cm diameter by 0.2 cm thickness. See two stainless steel disks at bottom of photograph.

Figure 6. Schematic drawing of containment can and arrangement for affixing can to uranium shielding plug.

tory hot laboratories following a 3.8 year activation. Unfortunately a small amount of cooling water had penetrated the holder, causing excess corrosion of the disks. The individual pieces had been plated with a nickel plating which did not hold up.

After activation the disks were encapsulated in a stainless steel can. Figure 6 is a schematic drawing of the can and the arrangement for affixing it to a uranium shield plug for insertion in our cobalt therapy unit. To insure good containment of the cobalt, a stainless steel plug was press-fitted over the last piece of cobalt. In addition, this joint was sealed with solder. Figure 7 shows the solder before it was melted; Figure 8, the joint after soldering; and Figure 9, source affixed to the plug. The source has been in daily use (except on Sundays) for three years without any sign of capsule failure, thus indicating that the method of final encapsulation has been successful.

Calculations following completion of source activation showed that the effective flux in the reactor at the locations of the cobalt disks had been 1.04×10^{14} neutrons per cm^2 per second,

assuming continuous operation of the reactor (neglecting shutdown time for periodic refueling, etc.).

By a comparison method using an ion chamber, it was determined that the specific activity of this source reached a value of 296 curies per gram, or a total activity of 3460 curies, which is a conversion of 26 per cent of the atoms to cobalt-60. The useful output of the source meas-

Figure 7. Top view of containment can before solder was melted. Picture taken through periscope of Argonne National Laboratory hot cave.

Figure 8. Top view of containment can after solder was melted. Can is placed in hole of heated block.

ured 64 roentgens per minute at the center of rotation of the Argonne Cancer Research Hospital's therapy unit.⁷

Our measurements with the above sources showed the dose fall-off at the periphery of the beam to be only slightly less steep than that of our 2 Mev supervoltage x-ray therapy unit. The focal spot of this therapy unit is somewhat smaller than the diameter of our cobalt source. We therefore feel that we have reached a practical lower limit for the cross-sectional area required for therapy with a cobalt-60 source.

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Figure 9. Photograph of 3460 curie cobalt-60 source holder affixed to uranium shield plug.

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BENZENE EXPOSURE AND APLASTIC ANEMIA FOLLOWED
BY LEUKEMIA 15 YEARS LATER*

By

R. L. DeGowin

Atom bomb victims have developed leukemia from one to fourteen years or longer after exposure to irradiation.¹ With such a long induction period, clues to the elusive cause of leukemia may disappear years before the disease becomes manifest. Recognition that patients recovering from aplastic anemia may later develop leukemia gives one a chance to observe the so-called preleukemic period,²⁻⁴ and careful biochemical studies, and morphologic and clinical descriptions of this period may provide clues to the nature of the preleukemic phase before the anarchic leukemic state supervenes. We therefore continue to study a number of such patients, of which the following is an example:

The patient, a 41 year old white house painter, had been extensively exposed (about 13 years) to benzene and later developed aplastic anemia; both conditions are associated with an increased risk of leukemia.²⁻⁵ Blood counts obtained during fifteen years after recovery from the aplastic anemia revealed a persistent hematopathy which finally emerged as subacute myelocytic leukemia.

CASE REPORT

Aplastic Anemia. The patient entered Hines Veterans Administration Hospital on July 15, 1947, with complaints of anorexia, nausea, dark urine, light stools, fevers and weakness for one week.

His blood pressure was 130/90 mm Hg, his pulse 120 per minute. The skin and conjunctivae were icteric. There was gingivitis. The spleen, liver and kidneys were not enlarged, but right upper abdominal tenderness was present. Initial blood counts were: hemoglobin 14 gm per 100 ml, red blood count 4.98×10^6 per mm^3 , white blood count 4,500 per mm^3 (segmented neutrophils 59 per cent, small lymphocytes 38 per cent, monocytes 2 per cent, eosinophils 1 per cent) (Figure 1, Table 1). The urine was normal except that it contained bile. Direct serum bilirubin was 12.1 mg per cent, total 18.0 mg per cent. Alkaline phosphatase was 7.8 Bodansky units. Icterus index was 101.2.

Choline chloride, vitamin B and thiamin were given, but there was only slight improvement, and then the patient began bleeding from his gums. Blood counts were not changed much, but two weeks later (August 20, 1947) when the bleeding persisted, the hemoglobin was 10 gm per 100 ml, red blood count 3.6×10^6 per mm^3 , white blood count 1,300 per mm^3 (10 per cent of which were neutrophils and 90 per cent were lymphocytes). There were only 30,000 platelets per mm^3 . A bone marrow aspirate on August 21, 1947 was hypocellular. Defective maturation of both gran-

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Table 1
PERIPHERAL BLOOD COUNTS

Date	Hg (gm/100 ml)	Hct (%)	RBC (x 10 ⁶ /cu mm)	Retics (%)	WBC (per cu mm)	Differential						
						PMN	Ly	Mon (%)	Eos	Bas	Platelets	Immature Cells
Aplastic Anemia												
7-17-47	14.0	-	4.98	-	4,500	59	38	2	1	0	-	
8-20-47	10.0	-	3.60	-	1,300	10	90	-	-	-	30,000	
8-22-47	8.5	-	3.31	0.2	-	20	80	-	-	-	-	
12-23-47	11.0	-	3.36	-	3,800	56	44	-	-	-	120,000	
1-21-48	11.0	-	3.40	-	2,800	-	-	-	-	-	-	
5-17-48	13.0	-	4.57	-	4,500	-	-	-	-	-	-	
8-17-48	14.0	-	4.72	-	4,200	-	-	-	-	-	-	
12-13-48	14.0	-	4.49	-	4,500	56	44	-	-	-	-	
6-16-49	13.0	-	4.23	-	4,000	54	46	-	-	-	-	
Preleukemic Phase												
1-20-56	12.3	42	3.55	0.5	7,000	74	17	9	-	-	109,000	1% neutrophilic myelocyte 2% metamyelocytes 3 normoblasts per 100 WBC 1 normoblast per 100 WBC
9-11-56	12.4	-	4.16	-	6,000	-	-	-	-	-	-	
3-20-57	13.5	-	4.36	-	5,800	-	-	-	-	-	-	
2-12-58	11.5	-	3.77	-	5,200	-	-	-	-	-	-	
2- 7-59	12.0	-	3.88	-	5,800	-	-	-	-	-	-	
5- 2-59	-	-	-	-	10,900	64	34	1	1	-	-	
1-23-60	11.7	-	4.65	-	11,400	64	23	10	-	-	-	
2-10-60	13.0	41	4.27	-	16,400	80	15	5	-	-	86,900	
3- 4-61	11.2	-	-	-	2,100	-	-	-	-	-	-	
1-25-62	-	-	-	-	4,100	-	-	-	-	-	-	
Leukemia												
5-17-62	7.4	24	2.19	-	2,700	31	53	3	-	-	-	6% myeloblasts 1% metamyelocytes 1% promyelocytes 5% neutrophilic myelocytes 7 normoblasts per 100 WBC
5-25-62	5.9	20	2.05	5.7	2,100	23	67	-	-	-	15,400	1% myeloblasts 6% neutrophilic myelocytes 10% metamyelocytes 11 normoblasts per 100 WBC
7-27-62	7.4	24	-	-	7,000	54	20	9	-	-	6,000	
10-2-62	11.8	32	-	-	10,900	-	-	-	-	-	17,000	

ulocytic and erythrocytic series was evident. In the former, the number of myelocytes equalled that of metamyelocytes and polymorphonuclear leucocytes; the polychrome normoblast was the predominate cell in the latter. Few blasts of any sort were seen. Megakaryocytes were scarce,

Figure 1. The chart depicts hemoglobin, leukocyte and platelet counts from 1947 through 1962. The shaded areas at the top and bottom represent the ranges of normal hemoglobin and leukocyte values respectively. Key: Hemoglobin (gm/100 cc) ●—●—●, Leukocytes (10³/cu mm) ●—●—●—●, Platelets (10⁴/cu mm) ●—●—●—●.

but there were numerous lymphocytes, plasma cells and fat-containing macrophages. A trephine biopsy of the sternum on August 23, 1947 revealed an extremely hypocellular marrow (Figure 2). The majority of cells occupying a few small foci of reticular proliferation were non-granular mononuclears resembling large lymphocytes and plasma cells. A few isolated groups of two to three normoblasts and granulocytes were present. No tubercles were found. Dr. George V. LeRoy, the hematologist consultant, concluded that aplastic anemia had developed after exposure to benzene during convalescence from severe hepatocellular jaundice.

Following Dr. LeRoy's recommendations, the patient was given fourteen pints of whole blood during the next four weeks. Extract of yellow bone marrow (one ampule daily) was given intramuscularly for about ten weeks. Penicillin (30,000 units every three hours) was given during the first week. Bleeding from the gums stopped after the four weeks of blood transfusions. A bone marrow aspirate on October 4, 1947 provided evidence of early but not too successful marrow regeneration.* No nucleated erythrocytic cells were seen in the hypocellular marrow. Lympho-

*Dr. LeRoy's interpretation.

cytes and mononuclear cells described as the "monocytoid leucocytes of Downey" were predominant. Despite low blood counts, the patient seemed sufficiently recovered to be discharged on December 31, 1947 (Table 1).

Subsequently the patient was given liver and iron injections and examined periodically in the outpatient department. He felt well and had no recurrence of bleeding. Hemoglobin values reached

Figure 2. A section of bone marrow obtained by trephine biopsy of the sternum on August 23, 1947 is hypocellular. Magnification X 350.

the lowest limit of normal, but mild leukopenia was constant. Anemia and leukopenia were still present on the last blood counts obtained at Hines Hospital on June 16, 1949 (hemoglobin 13.0 gm per 100 ml, white blood count 4000 per mm³, Table 1).

Preleukemic Period. No more blood counts were available until 1956 when the patient came to the University of Chicago Clinics complaining of belching and lower abdominal cramps. Except for a scar over his sternum and a grade 1/6 apical systolic murmur, his examination was normal. Anemia and mild thrombocytopenia were present (Table 1). During the next four years, while the patient was treated with bland diet and phenobarbital for an irritable colon, a persistent anemia was apparent from periodic blood counts.

One neutrophilic myelocyte, two metamyelocytes and normoblasts were reported on the peripheral blood film on January 23, 1960. The bleeding time from an ear puncture extended be-

yond fifteen minutes. On the day of the patient's first University of Chicago Hospitals admission (February 9, 1960), the liver was palpable two finger-breadths below the right ribs, but the spleen was not palpable. The left ear was red and swollen. Anemia, thrombocytopenia and leukocytosis were present (Table 1). No immature cells were seen on several blood film examinations while the patient was in the hospital. Increased mature granulocytes accounted for the elevated myeloid-erythroid ratio noted on the section of bone marrow aspirate obtained on February 11, 1960. Cellularity, maturation, megakaryocytes and stained iron were all normal (Figure 3).

Figure 3. A section of sternal bone marrow obtained by needle biopsy on February 11, 1960 shows normal cellular maturation and megakaryocytes. Magnification X 350.

After his discharge, the patient returned to work every day as a drill-press operator. He seemed healthy, but admitted that he bruised with slight trauma and that spontaneous bruising occurred. Routine blood counts demonstrated leukopenia and anemia on March 4, 1961 (Table 1). Fourteen months later, a personal physician told the patient that he was severely anemic and recommended that he return to the University of Chicago Hospitals.

Leukemia. The patient told of recent nose bleeds on his second admission (May 17, 1962) and ecchymoses were found on his arms, chest and legs. The heart was slightly enlarged. The spleen was firm 3 cm below the left ribs. A non-tender firm liver extended 10 cm below the right ribs. Initial blood counts were: hemoglobin 7.4 gm per 100 ml, hematocrit 24 per cent, red

blood count 2.19×10^6 per mm^3 , white blood count 2,700 per mm^3 (segmented neutrophils 31 per cent, lymphocytes 53 per cent, monocytes 3 per cent, myeloblasts 6 per cent, promyelocytes 1 per cent, neutrophilic myelocytes 5 per cent, metamyelocytes 1 per cent). There were 7 normoblasts per 100 leukocytes. Subsequent counts: reticulocytes 5.7 per cent and platelets 15,400 per mm^3 . Sections of bone marrow aspirate obtained on May 22, 1962 contained hypercellular marrow in which the myeloid-erythroid ratio was increased 10 to 1. Arrest of maturation in the myeloid series was evident from the predominance of myeloblasts and promyelocytes and a paucity of metamyelocytes and segmented neutrophils. Normoblasts were present. Megakaryocytes were rare. Prussian blue stained iron was abundant. A serum protein electrophoretic pattern was devoid of evidence of protein abnormality including a "myeloma spike." A gastric aspirate contained hydrochloric acid. Plasma iron was 110 gamma per cent; unsaturated iron binding capacity was 150 gamma per cent. Plasma clearance of Fe^{59} was about twice as rapid as normal. Ninety per cent of the injected radioiron appeared in erythrocytes in three days in contrast to a normal range of 60 to 70 per cent in 7 days. Uric acid (enzymatic method) was 6.4 mg per cent. Type A hemoglobin was detected by electrophoresis.

The patient was afebrile, and except for several days of bleeding from his sternal puncture, he felt moderately well. He received three units of blood, and because of thrombocytopenia and probable hemolytic anemia, prednisone 30 mg daily was begun several days before he left the hospital. The hematologists considered subacute myelogenous leukemia the most likely diagnosis.

The patient returned to his work taking prednisone 30 mg daily. He experienced easy bruising and more frequent night sweats, and on July 27, 1962, the discovery of recurrent anemia (hemoglobin 7.4 gm per cent) and thrombocytopenia (6,000 platelets per mm^3) prompted readmission to the hospital. Immature cells were seen in the peripheral blood. Petechiae were present in the skin and mucus membranes. The spleen was palpable 5 cm below the costal margin, and the liver extended 6 cm below the right ribs. Sternal bone marrow aspiration was performed on July 30, 1962, and sections were prepared. Sheets of immature cells with pale-staining nuclei resembling the myeloblasts of the previous sections occupied 90 per cent of the marrow (Figure 4). No mature leukocytes were seen (leukemic hiatus). Normoblasts were diminished, and megakaryocytes were rarely found. The marrow was unquestionably leukemic. The patient was transfused with three units of blood and discharged with a diagnosis of acute myelogenous leukemia. Blood counts in the outpatient department have shown persistent anemia and thrombocytopenia (Table 1).

DISCUSSION

Extensive exposure to benzene preceded a persistent hematologic abnormality starting with, as far as we know, a hypocellular bone marrow and pancytopenia which was followed by a nearly normal bone marrow with variable leukopenia, anemia and thrombocytopenia. Finally after fifteen years, a frankly leukemic marrow and pancytopenia were found. It is tempting to relate the blood diseases, or disease, to benzene exposure.

Benzene poisoning is not an invariable sequela of chronic benzene exposure,⁶ but when poisoning has occurred, the bone marrow varies from severe hypoplasia to extreme hyperplasia.⁷ The first signs of poisoning may appear with an infection long after exposure has stopped.⁵ Our patient's hepatitis may have resulted from either benzene or a virus.

Acute leukemia has occurred after benzene poisoning in several instances;⁶⁻⁸ one patient

Figure 4. A section of sternal bone marrow obtained by needle biopsy on July 30, 1962 is frankly leukemic. Sheets of myeloblasts replace normal marrow cells except for some normoblasts. Magnification X 375.

died with acute leukemia after twenty-three years of exposure to benzene.⁶ Furthermore, leukemia has developed in patients who have previously had a hypocellular bone marrow.^{2-4,9}

The duration of a preleukemic phase in our patient cannot be determined because no blood counts are available from June 1949 until January 1956. From 1956 until May 1962 (when the diagnosis of leukemia was made) his course was characteristic of preleukemia as described by Block, Jacobson and Bethard.² There was evidence of a hemorrhagic tendency, and thrombocytopenia was present. Leukopenia and anemia were also present, although not always simultaneously. In describing a patient who developed leukemia nine years after the beginning of a prolonged hematologic disorder, Williams observed an initially hypocellular marrow, a preleukemic period characterized by anemia, neutropenia and thrombocytopenia, and finally, the subsequent development of a hyperplastic marrow and myeloblastic leukemia.⁹ Although the maximal risk of leukemia occurred four to seven years after the atomic explosion in Japan, its incidence there was decidedly increased in exposed persons fourteen years after the blast;¹ a latent period similar to that described in this study. The leukemia rate in Japan ordinarily is twenty to thirty cases per million people each year. The 1958 leukemia rate in persons exposed within a 1,500 meter radius of the blasts in Hiroshima and Nagasaki exceeded 450 cases per million.¹

If benzene poisoning caused this patient's blood disease, one might speculate at great length

concerning the manner in which the delicate control of stem cell proliferation and maturation was perverted. As recognition of the preleukemic phase becomes more assured, the study of stem cell kinetics during that period will undoubtedly prove more rewarding than are present attempts to identify the cause of a leukemia during its end stages.

ACKNOWLEDGMENTS

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PROTECTIVE EFFECT OF AET ON THE IMMUNE MECHANISM OF X-IRRADIATED MICE*

By

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It is now well established that S,2-aminoethylisothiuronium (AET) protects the total mammalian organism against acute radiation death from dosages well above the lethal range.¹ Whether this is accomplished by preventing the death of cells or by some stimulation that facilitates more rapid proliferation of those cells which survive the initial damage, continues to be investigated.^{2,3} In addition, the question has been raised whether AET enhances the radiation survival of all kinds of cells. Thus Cudkowicz^{4,5} finds that AET does not protect mature immunologically competent cells of the mouse bone marrow, spleen, or liver against low doses of x-radiation. In similar experiments by Adachi and colleagues,⁶ however, pretreatment of homologous donor cells with AET failed to prevent secondary disease.

It seemed of interest to explore other systems to see whether pretreatment with AET would alter the loss of immune defenses against the invasion of tumor cells in resistant hosts. CF No. 1 mice are normally resistant to transplanted P-1534 lymphatic leukemia of the DBA/2 mouse, but when their immune mechanism is depressed by low dosages of x-rays (400 to 500 r), the leukemia will "take" and death will result. The proposed test involved pretreatment with AET before irradiation, followed by leukemic challenge, to see if AET would prevent death from the leukemia.

MATERIALS AND METHODS

The irradiation facility used was a G. E. Maxitron-250, operated at 250 kvp and 30 ma, with 0.25 mm Cu and 1.0 mm Al filtration.

CF No. 1 mice were obtained from Carworth, Inc., New City, N. Y. Supplies of DBA/2 mice, as well as the original inoculum of lymphatic leukemia P-1534, were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. This tumor can be passaged in the solid state by subcutaneous implant using a trochar, or the spleen may be ground gently in mortar and pestle and such cells injected i.p.

AET was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y., as was Thiogel-A, a thiolated gelatin used as a vehicle for oral administration of AET. Dosages of 9 mg of AET per mouse were administered i.p. in 0.2 ml isotonic saline, or introduced by stomach tube in 1.0 ml Thiogel-A as previously described by Laterjet *et al.*⁷

Groups of CF No. 1 female mice were x-irradiated with dosages ranging from 300 r to 900 r, with or without AET given i.p. or orally before exposure. They were then challenged either

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24 hours or 7 days later with spleen cells from leukemic DBA/2 mice. In one set of experiments the groups were given a weak challenge of only 100 cells per mouse. In other experiments, each group received a strong challenge of 50×10^6 cells per mouse.

In order to review the basic mortality curve for mice of this strain groups of CF No. 1 female mice were irradiated with dosages ranging from 550 r to 1400 r with or without AET orally or intraperitoneally before exposure. When AET was given intraperitoneally, the dose reduction factor was 1.7, while oral administration of a Thiogel-AET mixture protected only by a factor of 1.2 (Figure 1).

Figure 1. 30-day survival in CF No. 1 female mice.

RESULTS

Tables 1, 2 and 3 show that when irradiated CF No. 1 mice were inoculated with DBA/2 leukemia, survival occurred at higher irradiation levels in all groups of mice pretreated with AET, than in control irradiated mice. Results indicate that AET enhanced the ability of the immune mechanism to reject successfully the invasion of leukemoid cells, despite the irradiation doses administered. AET was more effective when given intraperitoneally than orally. With both modes of administration the dose reduction factors for leukemic deaths were comparable to the dose reduction factors obtained from mortality curves of AET-protected mice not challenged with leukemia.

Further experiments must now follow to elucidate whether the rejection of invading leukemoid cells was accomplished predominantly by original cells which survived treatment, or by the descendants of original cells.

Table 1

SURVIVAL OF IRRADIATED CF NO. 1 MICE INOCULATED
WITH 100 CELLS OF DBA/2 P-1534 LEUKEMIA

	Survival (%) following injection of 100 leukemic cells given			
	24 hrs. after x-ray		7 days after x-ray	
	x-ray only	AET i.p.	x-ray only	AET i.p.
300 r	100	-	-	-
400 r	37	100	-	-
500 r	20	77	12.5	100
600 r	0	87	0	100
800 r	-	50	-	50
900 r	-	40	-	50

Table 2

SURVIVAL OF IRRADIATED CF NO. 1 MICE INOCULATED
WITH 50 MILLION CELLS OF DBA/2 P-1534 LEUKEMIA

	Survival (%) following 50 million leukemic cells			
	24 hrs. after x-ray		7 days after x-ray	
	x-ray only	AET i.p.	x-ray only	AET i.p.
300 r	100	-	87	-
400 r	25	-	62	87
500 r	0	63	0	87
600 r	-	57	-	85
700 r	-	38	-	25
800 r	-	0	-	0

Table 3

SURVIVAL OF IRRADIATED CF NO. 1 MICE PRETREATED WITH
AET ORALLY AND INOCULATED WITH P-1534 LEUKEMIA

	Survival (%) following 24 hr. challenge			
	100 cells		50 million cells	
	x-ray only	AET orally	x-ray only	AET orally
300 r	-	-	100	-
400 r	38	100	25	25
500 r	25	25	0	38
600 r	0	25	-	8
700 r	-	12.5	-	0

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CYSTEAMINE PROTECTION OF X-RAY INDUCED
DYSPLASIA IN MOUSE HAIR*

By

F. D. Malkinson,[†] M. L. Griem, D. M. Phillips, and P. H. Morse

Profound morphological changes in the matrix and shaft of actively growing animal and human hair can be induced by irradiation¹⁻³ or by the administration of certain chemical agents.⁴⁻⁶ These induced changes may be remarkably similar in both forms of injury, and the number of hairs affected is proportional to the dose of radiation or chemical agent received. Recent investigations have revealed the usefulness of hair dysplasia in the mouse as a quantitative biological system for the demonstration of synergistic effects following the combined use of colchicine,^{7,8} L-triiodothyronine,⁹ or Actinomycin D,¹⁰ and radiation. For observations on other parameters of x-ray induced changes in hair—especially depigmentation—the reader is referred to the reports by Chase.¹¹⁻¹³

The present study was undertaken to determine the value of the hair dysplasia technique in assessing the efficacy of cysteamine (MEA) as a representative compound of established radio-protective potency.^{14,15}

METHOD

The studies were carried out on young adult female mice of the Carworth Farms #1 strain. Hairs in the resting phase of the growing cycle (telogen) are mitotically inactive and hence relatively insensitive to irradiation.² Plucking of telogen hairs immediately induces the mitotically active phase of hair growth (anagen), which is the radiosensitive stage of the cycle lasting 17-20 days in this strain of mouse.

At the beginning of each experiment hairs were plucked from the right haunch of all animals. Ten days later, experimental groups of 5 mice were given varying doses of radiation in conjunction with a constant amount of cysteamine. Two series of control groups were run: one series in which the animals received cysteamine alone and the other radiation alone. Four days later, the newly growing hairs were plucked from the prepared sites and examined microscopically as previously described.¹⁰

Following irradiation, the cages contained mice from different experimental groups, in an attempt to eliminate "cage" effects.

Previous studies⁷ have shown that the incidence of dysplasia produced in hairs at different dosages of radiation follows a reproducible sigmoid curve. As in the earlier studies, radiation was administered with a Machlett OEG-60 tube operating at 50 kv and 30 ma with 2 mm of aluminum added filtration. This produces 387r per minute surface dose at a focal skin distance of 11 cm and a beam quality of 1.2 cm half value depth in tissue. The animals were adequately

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shielded and treated individually without anesthesia. Surface doses ranged from 200r to 1600r increasing by increments of 100 or 200 r.

Five mgm of cysteamine hydrochloride (200 mgm/kg) was given intravenously as a freshly prepared solution in 0.5 cc of physiological saline. This was administered 45 minutes before, 10 minutes before, or immediately after irradiation. Initial observations revealed that cysteamine alone produced no significant hair dysplasia in dosages ranging from 2 to 10 mgm per animal, (80 - 400 mgm/kg).

Irradiated controls were given intraperitoneal injections of 0.5 cc of physiological saline.

All experimental data have been statistically analyzed using second order analysis of variance.¹⁶

RESULTS

The results of several separate experiments are shown in the accompanying figures where each point on a graph represents the average percent of dysplastic hairs from a single group of 5 mice.

Clear-cut protective effects of cysteamine on x-ray induced dysplasia are demonstrable throughout the dosage range of 300-900 r. (Figures 1-3.) The average dose reduction factor is about 1.5, and it appears to make little difference whether drug administration precedes irradiation by 10 minutes or by 45 minutes. No significant protection is seen with dosages of 1200r or higher (Figure 4).

Figure 5 shows that cysteamine fails to confer any protective effect on hair dysplasia when administered immediately after irradiation. Unexpectedly, some potentiation of hair damage by the drug actually occurs in the lower dosage ranges up to 600r ($P < 0.025$). This result has been confirmed in repeated experiments, and is being studied further.

DISCUSSION

The usefulness and sensitivity of the hair indicator technique for assessing protection against the damaging effects of irradiation undoubtedly reflect the high rate of cellular mitosis in the mouse hair matrix, where each epithelial cell divides once every 13 hours.¹⁷ Since the required rate of cellular replacement is high, damage to relatively few cells is apparently sufficient to produce microscopic morphological abnormalities. In these studies, the few days of the growth period remaining after irradiation were evidently insufficient to permit significant recovery from dysplasia in both cysteamine-protected and unprotected animals. This observation and the striking difference in the incidence of dysplastic hairs in drug-treated and untreated animals seen as early as 48 hours after irradiation suggest that the protective action of cysteamine here lies more in a reduction of the primary irradiation effects than in an acceleration of the recovery processes. However, radiation damage and possible enhanced recovery during the first 48 hours after exposure would not be detectable by this technique.

Previous investigations of protection against hair damage from radiation¹⁸⁻²² have been based solely on observations of subsequent retention or loss of hair. While this method permits qualitative evaluation of a protective agent—provided anagen has been previously induced—it does not permit quantitative conclusions. Slight or even moderate damage to anagen hairs is often followed by continued growth and will remain undetected by this gross technique. In our current studies, for example, there was no obvious loss of hair despite an incidence of up to

Figures 1 and 2. Lowered incidence of hair dysplasia over a wide dosage range after irradiation of mice protected with 5 mgm of cysteamine (MEA).

Figure 3. Lowered incidence of hair dysplasia over a wide dosage range, showing significant differences between pre- and immediate post-treatment administration of cysteamine in irradiated animals. Post-treatment cysteamine fails to protect against hair dysplasia (see also Figure 5).

Figure 4. Failure of pre-treatment cysteamine to protect against hair dysplasia in the high x-ray dosage ranges.

Figure 5. Moderate potentiation of hair dysplasia in the lower x-ray dosage ranges following cysteamine administration immediately after irradiation.

40 per cent dysplasia with X-ray doses as high as 500r. Only at and above dosage levels of 600r was slower growth apparent and alopecia noticeable. In the cysteamine-protected animals alopecia was not seen at dosage levels of less than 800r, although dysplastic hairs were found after as low a dose as 300r.

The degree of radio protection afforded anagen hairs by cysteamine compares favorably with the dose reduction factor of 1.8 for lethal radiation in mice established previously by other investigators.²³ The reliability, simplicity and directness of the technique herein described make it generally useful in the evaluation of potential radioprotective agents.

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PREVENTION OF DEATH IN MICE AFTER LETHAL IRRADIATION OF THE HEAD*

By

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Several species of laboratory animals die 9 to 14 days after irradiation of the head with 2000 to 4000 r. Such death is preceded by a progressive weight loss starting about 5 days after irradiation, and has been termed by Quastler "oral radiation death."¹ Previous studies on mice in this laboratory have indicated that starvation and dehydration play a major role in such deaths,² and are accompanied by changes in the mucosa of the tongue and soft palate, loss of weight, and marked decrease in water intake and urine output. Weight loss was progressive, amounting to about 39 per cent at death 9 to 11 days after irradiation. The changes in the mucosa progressed to frank ulceration about 8 to 9 days after irradiation, and apparently interfered with the normal ingestion of food and water. It appeared that if nutrition and hydration could be maintained in some manner other than the normal way of feeding, death might be prevented. The present study was undertaken to investigate the effects of liquid diets, parenteral feedings, and gastrostomy tube feedings.

MATERIALS AND METHODS

Male C57 B1 mice, anesthetized with Nembutal, were placed in lead shields which permitted irradiation of the head only, and given radiation at 250 kv with a standard therapy machine.[‡] Details of the radiation and shielding technique have been described previously.²

Various methods were attempted to maintain adequate nutrition. Some mice were fed a liquid diet of equal volumes of evaporated milk and water with the addition of 40 gm per liter of glucose. Others were given subcutaneous injections of a solution of 5 per cent glucose and 0.9 per cent NaCl, aminosol,^{**} or a mixture of penicillin and streptomycin.^{††}

Other mice were fed by means of indwelling gastrostomy tubes (Figure 1), of polyethylene tubing, ID 0.055 inches and OD 0.075 inches. The tube was heat fashioned over a bunsen burner and spun on a wire lathe to produce the flanges. One end of the tube was inserted in the squamous portion of the stomach, and the gastric wall was sutured between the flanges. The mid-portion of the tube was bent to lessen tension on the gastric wall. The tube was brought through the muscular layer of the abdominal wall, and subcutaneously to the back of the neck where it was passed through the skin and the large terminal flange sutured. A toothpick was used to close the external end of the tube between feedings. The mice were caged separately to prevent

*This report is taken from a paper which will appear in Radiation Research.

[†]Walter G. Zoller Memorial Dental Clinic, and the Department of Pathology, University of Chicago.

[‡]G. E. Maxitron.

^{**}A hydrolized protein made by Abbott Company.

^{††}Combiotic made by Pfizer Company.

damage to the tubes. One ml of a liquid diet was injected through the tube every hour for 12 hours a day from the 5th to the 20th day after irradiation. The diet was prepared to satisfy the

Figure 1. The polyethylene gastrostomy tube. At the left is the end which was placed in the squamous portion of the stomach. At the right is the external end of the tube which was kept closed between feedings.

nutritional requirements of mice described by Morris,³ and was a modification of that employed by Moskowitz.⁴ It consisted of defatted, dehydrated, eggs 32 per cent; vitamin-dextrin mix 10.8 per cent, dextrin 10.2 per cent; alphacel 6.0 per cent; salts 4.0 per cent and corn oil 37 per cent. An equal volume of water was added to make the consistency satisfactory for gastrostomy feeding.

RESULTS

Twenty-four mice irradiated with 2000 r to the head were given the sweetened milk diet starting the 5th day after irradiation. The survival rate 20 days after irradiation was approximately 18 per cent, and the peak incidence of death was 1-1/2 days later than that of non-treated, irradiated controls. The weight loss in the surviving mice was nearly as great as in those that died. The surviving mice appeared sick until about the 18th day after irradiation after which time their weight increased. After the 20th day they seemed nearly normal. Of 12 mice similarly irradiated and fed a chow and water diet ad libitum, there were no survivors after the 11th post-irradiation day.

In a group of mice receiving 4000 r to the head and fed the sweetened milk diet, there were no survivors and there was no difference in the time of peak incidence of death as compared to controls.

In another group of 12 mice irradiated with 2000 r to the head, receiving subcutaneous injections of 2 ml of a solution of 5 per cent glucose in 0.9 per cent NaCl 8 times a day starting the 5th day after irradiation, the survival rate was about 18 per cent. The behavior of the mice during the experiment was similar to that of the mice on the milk diet.

Other mice given 2000 r to the head were treated with subcutaneous injections of aminosol, 2 ml 8 times per day; and others similarly irradiated were given 2 ml subcutaneous injections of a mixture of penicillin and streptomycin 8 times per day for a total dose of 8000 U of penicillin and 5000 μ g of streptomycin per day. In all cases weight loss was similar to that of non-

treated irradiated control mice and the survival rate was not more than 18 per cent. There were never any survivors in control groups of 12 mice irradiated and kept on a chow and water diet.

Although a few of these mice survived a dose of radiation which was lethal to controls, it was evident, even in the survivors, that replacement of fluid and nutritional requirements was inadequate. Attempts were therefore made to accomplish feeding by means of an indwelling gastrostomy tube.

To determine whether mice could be maintained adequately by gastrostomy feeding, normal mice were fed by this method alone for 15 days. The mortality rates ranged from 60 to 94 per cent, but in individual survivors, the weight loss was never more than 23 per cent. All dead mice were autopsied and the reasons for gastrostomy failure were found to be: the tube came out of the stomach, became plugged, or developed a leak allowing the diet mix to spread under the skin or into the peritoneal cavity. The cause of death could usually be predicted. When the tubes were plugged, the animals lost weight and died. In the case of a tube leak, the mouse often gained weight, the abdomen became distended, the skin was fixed to the underlying structures, and food leaked out around the external end of the tube. After about 12 days of tube feeding, subcutaneous abscesses often developed at the external site of the tube although the tube was intact and functioning.

The survival rate of normal mice fed by gastrostomy tube could be maintained at 40 per cent by certain technical improvements, such as: bending the mid-portion of the tube near the internal end to lessen tension on the stomach wall; double suturing of the stomach wall around the internal end of the tube; the use of blunted needles for injecting the diet mix into the tube; the use of a reamer to unplug tubes; injecting no more than 1.0 ml of diet mix at one time; and by using a special mouse holder during feeding in order to control sudden movements which tended to pull the tube loose. The subcutaneous abscesses were treated by incision and drainage, and irrigation with an antibiotic solution.*

In all, 101 gastrostomized mice were irradiated with 2000 r to the head and were fed the diet mix through the gastrostomy tubes from the 5th day after irradiation until the 20th day, or until death. The experience with non-irradiated controls indicated that it was possible to establish with reasonable certainty whether death was due to technical difficulties with the tubes. All animals dying after irradiation were autopsied and the functional state of the gastrostomy was determined. If deaths due to gastrostomy failure were excluded, the survival rate after 2000 r head irradiation was 94 per cent (Table 1). In these animals the weight loss averaged 21 per cent and never exceeded 25 per cent. If all deaths, including gastrostomy failures, are accepted, the survival rate was 40 per cent. Occasionally death occurred when the gastrostomy appeared intact, but such deaths did not exceed 6 per cent of the total group. At autopsy these mice were found to have pneumonia. Many of these deaths occurred before or after the usual survival time for "oral radiation death." By the 18th day after irradiation the surviving mice began to eat and drink again and by the 20th day the mice were able to maintain themselves in a normal way.

*Combiotic made by Pfizer Company.

Table 1
THE EFFECT OF GASTROSTOMY FEEDING ON
"ORAL RADIATION DEATH"

	No. of animals	% survival	% survival <u>excluding</u> <u>gastrostomy</u> <u>failures</u>
<u>Irradiated mice</u> <u>2000 r to the head</u>	24	0	-
<u>Irradiated mice</u> <u>2000 r to the head,</u> <u>gastrostomy fed</u>	101	40	94
<u>Normal mice</u> <u>gastrostomy fed</u>	39	41	100

DISCUSSION

Irradiation of the head of mice with 2000 r without post-irradiation nutritional treatment consistently produces 100 per cent mortality. Parenteral feedings resulted in the survival of some mice receiving 2000 r to the head, but did not supply all the nutritional requirements. The survival of a few such mice so fed and treated with antibiotic injections is more likely due to the administration of fluid than to the antibiotics, since earlier studies^{1,2} have demonstrated that infection does not play a major role in the mechanism of oral radiation death.

The feeding of a more complete diet through gastrostomy tubes resulted in a 94 per cent survival rate in mice receiving 2000 r to the head, thus indicating that mice can survive such lethal irradiation if nutrition and hydration are maintained. The surviving mice lost no more than 25 per cent of their original weight during the experiment. Normal mice handled frequently every day will have 10 to 15 per cent variation, usually a loss, in weight.

The observation that the mice begin to eat again about 18 days after irradiation implies that the lingual and palatal ulcerations, described previously,² must have healed sufficiently by that time to permit oral ingestion. Observation revealed that healing was evident about 13 to 14 days, and completed before or at least by 20 days after irradiation. Renewal of the tongue epithelium in the guinea pig was reported by Platt to take about 8.4 days.⁵ This is consistent with our observation that thinning of the lingual epithelium was evident about 5 days after irradiation, with frank ulceration occurring at 8 to 9 days. The lingual ulceration apparently forms because of the lack of re-population of the epithelial tissue. Toto reported the epithelial generation cycle in the mouse tongue to be 100 hours.⁶ Since healing was not evident until about 13 days after irradiation, the implication is that mitotic activity may be inhibited for about 9 days after irradiation in the epithelium of the posterior dorsum of the tongue.

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THE EFFECT OF VITAMIN E ON THE CARCINOGENICITY OF METHYLCHOLANTHRENE*

By

S. L. Haber[†] and R. W. Wissler

Wheat germ oil, which is very rich in alpha tocopherol (Vitamin E), is one of the dietary factors reported to affect the carcinogenic response. Jaffe¹ reduced the incidence of response to an intraperitoneal injection of methylcholanthrene more than 50 per cent by adding wheat germ oil to the diet of his mice. He did not believe that the results could be attributed to the alpha tocopherol, though this vitamin had been shown to reduce the toxic effects of methylcholanthrene. Haddow,² as well as Cameron and Meltzer,³ have concluded that alpha tocopherol does not lessen the incidence of tumors from skin paintings with carcinogens. Rowntree, *et al.*,⁴ fed a crude wheat germ oil to rats and guinea pigs without other carcinogens, and claimed to have produced sarcomas in the abdominal cavity with an induction period as short as 15 days. Others⁵⁻⁹ were unable to confirm these findings.

There is, however, apparently no previous investigation of the effect of alpha tocopherol on subcutaneously injected methylcholanthrene. The purpose of this study was to ascertain whether the oral administration of this vitamin exerted any effect on the production of sarcomas by methylcholanthrene.

EXPERIMENTAL DATA

In the first experiment, 140 female mice of the C57 Leaden strain were divided into two equal groups, one of which had 0.67 g of alpha tocopherol added to each kilo of its Laboratory Chow diet—an increase of almost 100 times the normal content. The critical subgroup in each major group received a subcutaneous depot of methylcholanthrene in mineral oil (0.1 mg/ml) in the interscapular region (Table 1).

None of the 70 control animals developed tumors in the 24 weeks of the experiment. Thirteen fibrosarcomas were found in the critical subgroup receiving a regular diet, while only 8 appeared in the critical subgroup fed a high vitamin E diet. This represents 54 per cent and 30 per cent, respectively, of those animals surviving after the appearance of the first tumor.

In the second experiment, 130 C57 Black male mice were divided into 2 equal groups, one of which had 7.0 g of alpha tocopherol added to each kilo of its Laboratory Chow diet. The two critical subgroups in each group received an interscapular subcutaneous depot of methylcholanthrene (0.1 mg/ml) dissolved either in tricaprylin or a solution of 10 per cent Tween 60 and 90 per cent tricaprylin (Table 2).

In 26 weeks, two lymphomas appeared in the control animals; an incidence of 6 per cent.

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The mice in the critical subgroups on a normal diet developed a total of 13 tumors (9 fibrosarcomas, 3 lymphomas, 1 hepatoma); an incidence of 33 per cent after correction for early deaths. The critical subgroups on a high vitamin E diet had a total of 9 tumors (7 fibrosarcomas, 1 lymphoma, 1 squamous carcinoma); a corrected incidence of 21 per cent.

Table 1

COMBINED EFFECT OF DIETARY VITAMIN E AND SUBCUTANEOUSLY INJECTED METHYLCHOLANTHRENE ON C57 LEADEN STRAIN FEMALE MICE

Group I - Normal diet		Group II - Normal diet + 100 x Normal amount of Vitamin E	
Sub-Group I-a	Sub-Group I-b	Sub-Group II-a	Sub-Group II-b
No methylcholanthrene	Injection of 0.1 mg methylcholanthrene*	No methylcholanthrene	Injection of 0.1 mg methylcholanthrene*
No tumors	54% survivors developed tumors	No tumors	30% survivors developed tumors

*Dissolved in 1 ml mineral oil.

Table 2

COMBINED EFFECT OF DIETARY VITAMIN E AND SUBCUTANEOUSLY INJECTED METHYLCHOLANTHRENE ON C57 BLACK STRAIN MALE MICE

Group I - Normal diet		Group II - Normal diet + 1000 x Normal amount of Vitamin E	
Sub-Group I-a	Sub-Group I-b	Sub-Group II-a	Sub-Group II-b
No methylcholanthrene	+ injection of 0.1 mg methylcholanthrene*	No methylcholanthrene	+ injection of 0.1 mg methylcholanthrene*
No tumors	33% survivors developed tumors	No tumors	21% survivors developed tumors

*Dissolved in 1 ml tricaprylic or 1 ml (10% Tween 60 and 90% tricaprylin).

DISCUSSION

C57 mice have a low incidence of spontaneous tumors, but respond uniformly well to carcinogens. Age does not seem to be a prominent factor, although more primitive sarcomas are produced in very young mice.¹⁰

Mineral oil and tricaprylin, if critically purified, apparently have little or no specific influence on carcinogenesis when used as a vehicle in a subcutaneous depot.

Alpha tocopherol appreciably inhibited the carcinogenic activity of methylcholanthrene in the first experiment, and failed to itself produce tumors in the controls.

In the second experiment, which had a basically similar plan, although the mice differed in sex and substrain, dosage of vitamin E, and vehicle used for the carcinogen, inhibition of the

carcinogenic activity of methylcholanthrene by alpha tocopherol was confirmed.

The significance of these results approximates the 93 per cent level.^{11,12}

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RELATION BETWEEN THE ABSORBANCY AND THE QUENCHING
OF LIQUID SCINTILLATION SAMPLES*

By

J. De Bersaques[†]

In all work with radioisotopes an accurate knowledge of the efficiency of the counter is essential. It is even more important in liquid scintillation counting of biological materials which may introduce complex quenching possibilities.

The standard method of assessing the efficiency for each sample is to add an internal standard of known activity of the same isotope to the sample and then count the "loaded" sample. From the difference between the two counts the efficiency can be calculated simply.

In the course of a work on labeled amino acids in hairs,¹ an unpredictable intensity of brown color was formed in some samples when the hairs were left to dissolve in Hyamine X-10 (24 hours at 55° C). Because of the large number of samples involved, a simple way of measuring the efficiencies was sought. Curves such as those of Hayes² and Toporek³ could not be used because neither the color nor the quenching were related to the weight of hair in a sample.

Developing an idea of Davidson,⁴ who noted that quenching produced a shift in pulse height, Baillie⁵ and Takahashi⁶ used a calculation in which the quenching is plotted from the ratio of the counts in two parts of the spectrum. This method, however, is not usable with tritium, because quenching produces only a minimal pulse height shift in this very weak β emitter. The calculation is also inapplicable when the sample contains two different isotopes (e.g., H^3 and C^{14} or H^3 and S^{35}) and both scalers are used to count them simultaneously.⁷

The presence of a colored compound led us to inquire whether the quenching could be related to the optical density of the solution. The wave length of 430 m μ was chosen as a compromise between the maximal emission of the scintillators and the sensitivity peak of the photomultipliers.⁸

The measurements were done with a Tri-Carb model 314 A spectrometer. The counting solution contained 0.3 to 1 mg of dissolved guinea pig hair, 3 g/l PPO and 0.1 g/l POPOP in toluene; its volume was 15 ml of which 0.5 ml was Hyamine X-10 1 M in methanol. H^3 -leucine and S^{35} -cystine were the labeled substances used; the settings of the windows were 7.5-25 V and 7.5-80 V at 1325 V, and 10-34.5 V and 10-100 V at 1090 V respectively.

The optical density was measured in the 25-mm tubes of a Coleman Junior spectrophotometer with counting solution but without hair as a blank. Direct measurement in the counting vials with a Beckman DU spectrophotometer was attempted but did not give as reliable results.

The relation between quenching and optical density was found to be linear up to approximately 50 per cent quenching. In Figure 1 the number of c.p.m. due to an added internal sample

*This report is taken from a paper which appears in The International Journal of Applied Radiation and Isotopes. The work was done while the author was "Aspirant du Fonds National de la Recherche Scientifique," Belgium and International Postdoctoral Fellow of the National Institutes of Health.

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Figure 1. Relation between the optical density and the number of counts per minute due to an added internal sample of H³-leucine in 25 experiments and regression line.

Figure 2. Relation between the optical density and the number of counts per minute due to an added internal sample of S³⁵-cystine in 25 experiments and regression line.

of H^3 -leucine is plotted against optical density and the regression line of the 25 experiments is drawn. The correlation coefficient of this line is -0.983 and the standard error of the estimate 110 c.p.m.

Figure 2 gives a similar graph for S^{35} -cystine. The correlation coefficient here is -0.953 and the standard error of the estimate 226 c.p.m. If the ratio method of Baillie⁵ is used for the same samples, the correlation coefficient is -0.970 and the standard error 182 c.p.m.

This method is evidently only applicable with coloured quenchers and its validity is best tested for each particular system under study.

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THE NON-ANTIGENICITY OF SYNTHETIC POLYRIBONUCLEOTIDES AND APURINIC ACID*

By

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Much of the information available on the antigenicity of nucleic acids is clouded by the fact that the materials used were contaminated with protein and other cellular constituents so that the specificity of the antigen-antibody reactions observed has been unclear.¹ Recently, however, two facts have emerged which leave little doubt that nucleic acids can participate in immunologic reactions. The first is the observation that T₂, T₄, and T₆ phage DNAs, when highly purified, are capable of interacting with antisera prepared by inoculation of animals with whole phage lysates.² The antigenicity of these DNAs depends upon the presence of glucosylated 5-hydroxymethylcytosine.^{3,4} Second, the sera of certain patients suffering from lupus erythematosus contain antibodies capable of reacting with native or denatured DNAs of diverse source.^{5,6} The antigenic site for this interaction appears to involve regions of the DNA molecule where thymine occurs in repetitive sequence.⁷

In further exploration of the problem of the antigenicity of polynucleotides, attempts were made to immunize rabbits with the synthetic polynucleotides polyadenylic, polyuridylic, polycytidylic, and polyinosinic acids. These homopolymers were prepared from the appropriate nucleoside diphosphates using a polynucleotide phosphorylase derived from *M. lysodeikticus*⁸ and were virtually protein free. In addition, attempts were made to immunize rabbits with apurinic acid⁹ derived from commercially obtained beef thymus DNA (Sigma Chemical Co.). Two animals were used for each polynucleotide. An initial injection of material emulsified in complete Freund's adjuvant was followed by 3 weekly series of 3 injections in saline, each animal receiving a total of from 7.5 to 10 mg of polynucleotide. The animals were bled 7 days after the last injection, and the sera were examined for the presence of antibody by the following methods: Ouchterlony double diffusion in agar gel, Oudin single diffusion in agar gel, complement fixation, and classical immunoprecipitin techniques. In all cases the results were entirely negative.

Synthetic homopolynucleotides were used in this experiment because they do not occur in nature, and it was hoped that, like certain synthetic polypeptides, they would have antigenic properties. Highly purified T₂ phage DNA has been found incapable of producing antibodies when used as the immunizing material *per se*.¹⁰ Methods are therefore being sought of combining polynucleotides with effective protein antigens such as bovine serum albumin in the hope that such a material will be effective in eliciting an antibody capable of interacting with the pure polynucleotides.

*Summary of a paper which appears in *Nature*, 195:1319, 1962.

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THE ENZYMATIC SYNTHESIS OF 5-RIBOSYLURIDYLIC ACID*

By

R. L. Heinrikson† and E. Goldwasser

Recent investigations into the metabolic origin of the carbon-carbon ribosyl linkage in 5-ribosyluracil and its phosphorylated derivatives have suggested that these compounds may arise either by a mutase type of reaction,^{1,2} or by the rearrangement of a uridine derivative.³ We have previously described a crude extract from the pyrimidine-requiring protozoan *Tetrahymena pyriformis* which catalyzes the conversion of uracil to both UMP and 5-ribosyluridylic acid, utilizing PP-ribose-P as ribose source.⁴ The evidence now indicates that whereas UMP is formed by way of the conventional pyrophosphorylase mechanism,⁵ the ribose moiety of Ψ UMP† is not derived directly from PP-ribose-P. The data presented below suggest the existence of a new pathway of nucleotide biosynthesis and provide the first detailed account of the enzymatic formation of the 5' nucleotide of 5-ribosyluracil.

Tetrahymena pyriformis (Strain W) was grown under sterile conditions on 2 per cent proteose peptone (DIFCO) without aeration. Cells from six liters of medium were harvested after 4-5 days growth and were washed twice with cold distilled water. All subsequent steps were carried out at 4-6° unless otherwise noted.

The washed cells were collected and quickly frozen. This procedure routinely yielded 10-15 cc of packed cells. The pellet was then ground with twice its volume of washed glass powder** in a mortar previously cooled to -20°. The broken cells were extracted twice with 60 ml portions of 0.01M tris, pH 8.0, containing 20 per cent glycerol and the combined extracts centrifuged for 2 hours at 100,000 g. The 100,000 g supernatant solution was fractionated with ammonium sulfate and that fraction which precipitated between 60 and 90 per cent saturation†† was collected and dissolved in 0.01M tris pH 8.0, containing 20 per cent glycerol. This solution was then dialyzed 16 hours against two 100 volume changes of buffer.

A solution of the dialyzed 60-90 per cent ammonium sulfate fraction containing approximately 50 mg of protein was further fractionated on an ECTEOLA cellulose column (7 cm x 1 cm²). After extensive washing, the column was eluted in stepwise fashion with 0.05M tris (fraction E-II) and 0.10M tris (fraction E-III), both buffers at pH 8.0 and containing 20 per cent glycerol. These enzyme fractions could be stored two months at -20° in the presence of glycerol without loss of activity.

The various enzyme fractions were assayed as described in Table 1. This assay is based on the conversion of uracil to the 5' nucleotides, UMP and/or Ψ UMP. The incubation mixtures

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‡ The abbreviation used is: Ψ UMP, 5-ribosyluridylic acid.

** Superbrite, Minnesota Mining and Manufacturing, Catalog No. 100.

†† This is based on the assumption that in the tris-glycerol system employed, at 4° C, saturation with ammonium sulfate is reached at a concentration of 500 g per liter.

were boiled to terminate reaction, and the filtrates subjected to chromatography on columns of Dowex-1-formate-X-8 ($0.78 \text{ cm}^2 \times 20 \text{ cm}$). Columns were routinely washed with 10-15 volumes of water and then with 30-40 volumes of 0.2 M formic acid. The $5' \Psi \text{UMP}$ and $5' \text{UMP}$ were then eluted in that order with 0.05 M ammonium formate + 0.005 M formic acid, pH 4.6. The ΨUMP obtained was concentrated as described by Cohn⁶ and identified with respect to its spectral characteristics at neutral and elevated pH⁶ and by paper chromatography in ethanol - 0.5 M ammonium acetate (5:2, volume for volume) pH 3.8.⁷ Hydrolysis of the enzymically formed $5' \Psi \text{UMP}$ by crude snake venom yielded equimolar quantities of 2-C^{14} 5-ribosyluracil and orthophosphate.

Table 1

SYNTHESIS OF URACIL NUCLEOTIDE BY ECTEOLA FRACTIONS E-II AND E-III

The incubation mixtures (50 ml) contained 0.5 mM magnesium acetate; $6.5 \mu \text{M}$ 2-C^{14} uracil; $10 \mu \text{M}$ of either PP-ribose-P or Ribose-5-P and 10 ml of enzyme in the corresponding tris-glycerol system pH 8.0 containing 6.0 mg protein. Reactions were carried out at 30°C and were terminated after two hours. (See text.)

Experiment	Fraction	Total μM ΨUMP formed	Total μM UMP formed	% Total uracil nucleotide as	
				ΨUMP	UMP
A (PP-ribose-P)	E-II	0.02	0.41	4	96
	E-III	0.25	0.01	95	5
B (Ribose-5-P)	E-II	0.02	0	100	0
	E-III	0.53	0	100	0

In experiment A (Table 1) enzyme fractions E-II and E-III were incubated with 2-C^{14} uracil, Mg^{++} , and PP-ribose-P. Analysis of the incubation mixtures showed that of the total nucleotide formed in each case, fraction E-II gave rise primarily to UMP, while the main product of the reaction catalyzed by fraction E-III was ΨUMP . The enzymes responsible for the biosynthesis of these isomers have therefore been separated by the purification procedure reported. UMP pyrophosphorylase has been isolated from several systems,^{5,8} and this experiment suggested the presence of a comparable enzyme catalyzing the condensation of PP-ribose-P with uracil at C_5 rather than N_3 . However when these enzyme fractions were incubated in systems containing ribose-5-P* as ribose source (experiment B, Table 1), a two-fold stimulation in ΨUMP synthesis was observed as compared to the mixture containing PP-ribose-P (experiment A). The nucleotide product of these reactions was 100 per cent ΨUMP in each case; no UMP was detected. Nucleotide synthesis was shown to be absolutely dependent on the addition of either PP-ribose-P or ribose-5-P.

These experiments clearly indicate that in *Tetrahymena pyriformis* the formation of UMP is catalyzed by a UMP pyrophosphorylase that is entirely distinct both in its chemical properties and in its mode of action, from the enzyme (or enzymes) responsible for ΨUMP biosyn-

*The sodium salt was used: 98-100 per cent purity (Sigma).

thesis. The observation that Ψ UMP was formed in the presence of PP-ribose-P may be explained by the fact that this material (Pabst) contains significant amounts of ribose-5-P. This latter compound may also be produced during the course of the reaction by hydrolysis of the PP-ribose-P. The evidence presented excludes the possibility that Ψ UMP might be formed via a rearrangement of UMP and makes highly unlikely the possibility of a mutase mechanism at the nucleoside level. The addition of unlabelled uridine to these reaction mixtures had no effect on the specific radioactivity of the Ψ UMP formed, nor was a Ψ uridine* kinase demonstrable in the preparation. In the reaction catalyzed by fraction E-III, uracil was converted stoichiometrically to Ψ UMP, whereas there was a considerable loss of ribose-5-P.

Lis and Allen⁹ have recently reported the synthesis of nucleosides and nucleotides of isocytosine in a crude system in which ribose-5-P could serve as a ribose source, though it was less effective than PP-ribose-P. The data we have presented in this communication demonstrate that the formation of Ψ UMP by *Tetrahymena* preparations involves the condensation of uracil with ribose-5-P or some ribose derivative other than PP-ribose-P. Although it seems unlikely that the enzyme preparation contains appreciable amounts of nucleoside polyphosphate, we are unable to exclude the possibility that these compounds may be involved as cofactors or activators in the condensation. The observed loss of ribose-5-P may be coupled indirectly to the enzymatic synthesis of the carbon-carbon bond. Further investigations into the energy and cofactor requirements of the reaction and characterization of the enzyme are being pursued at present.

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*The abbreviation used is: Ψ uridine, 5-ribosyluracil.

PROPERTIES OF THE DNA-DEPENDENT SYNTHESIZED RNA

By

S. B. Weiss*

Enzymes which catalyze the polymerization of ribonucleotides under the direction of DNA have been identified in a variety of species.¹⁻⁷ Although we had initially demonstrated DNA-dependent synthesis of RNA in rat liver nuclei,⁶ the mammalian preparations did not lend themselves to extensive purification. Therefore, we directed our attention to the purification of an RNA polymerase found in extracts of *Micrococcus lysodeikticus* and have attempted various studies which would help define the mechanism of this unique polymerization reaction (Figure 1).

Figure 1. Reaction scheme for RNA polymerase.

The data accumulated so far suggest that the RNA product of this reaction is a faithful copy of the DNA primer, and, furthermore, that both strands of the DNA double helix function as templates resulting in the production of complementary RNA chains.

Until recently, the requirement of this enzyme for a deoxypolynucleotide primer appeared to be specific. However, experiments with highly purified preparations of RNA polymerase suggest that synthetic ribopolynucleotides can also prime the synthesis of RNA. The experimental data leading to these conclusions has been obtained from work carried out in collaboration with Mr. J. Moohr, Mr. F. Fox, Drs. T. Nakamoto and E. P. Geiduschek and shall be reviewed in the following discussion.

ANALYSIS OF REACTION PRODUCT

RNA polymerase activity may be readily detected by the incubation of bacterial extracts with the four ribonucleoside triphosphates, a divalent metal ion (either magnesium or manganese) and a DNA primer. If one of the ribonucleoside triphosphates is labeled with C^{14} , or with P^{32} in the innermost phosphate, the polymerization reaction can be followed by the incorporation of isotope into the acid-insoluble fraction. Most microbial extracts are sufficiently rich in

*Summary of a report which was presented at the Symposium on Informational Macromolecules, at Rutgers University, September 5-7, 1962, and will be published in the Proceedings of the Symposium.

RNA polymerase so that even with relatively crude preparations of enzyme it is possible to demonstrate, in vitro, the net accumulation of newly formed RNA.⁷ The equilibrium of the reaction is such that RNA synthesis is highly favored. Examination of the RNA formed has shown that the average base composition is nearly identical with that of the DNA primer.^{8,9} In the absence of adequate methods for determining the sequential arrangement of bases in polynucleotide chains, we examined the frequency with which the individual ribonucleotides were incorporated next to each other with respect to different primers. Here too the average nearest-neighbor frequency for the sixteen possible base-pairs of the enzymatically synthesized RNA is identical to the DNA primer employed.¹⁰ The results from these two independent experiments are consistent with the concept of RNA replication of its DNA template.

DNA-RNA COMPLEX FORMATION

The RNA assembled in vitro by RNA polymerase may be easily separated from DNA by CsCl density gradient centrifugation.¹¹ Thus, one may prepare net quantities of enzymatically formed RNA completely free of primer and starting reactants. Using T2 DNA as primer we have been able to prepare significant quantities of T2 RNA-P³² by this procedure. When T2 RNA-P³² was added to fresh T2 DNA, and this mixture heat-denatured and then held at 40° for several hours (annealing), DNA-RNA complexes readily formed. Under identical conditions, the annealing of T2 RNA-P³² with DNA's other than T2, did not yield hybrid formation of this kind.¹¹ Demonstration of the specific interaction of enzymatically prepared T2 RNA with T2 DNA suggested the presence of entire nucleotide sequences in both primer and product that were identical. A similar type of specificity for hybrid formation had been shown earlier by Hall and Spiegelman.¹²

SECONDARY STRUCTURE ANALYSIS

From the extent of DNA-RNA complex formed, we had tentatively concluded that both DNA strands must serve as template for complementary RNA synthesis.¹¹ This conclusion was subsequently corroborated by Chamberlin and Berg.¹³ We therefore considered the possibility that the enzymatically synthesized ribopolynucleotide consisted of two separate chains which could be self-complementary and under appropriate conditions exhibit physical properties similar to helical DNA. This possibility was confirmed when we were able to demonstrate that the synthetic RNA, isolated by the CsCl procedure, had secondary structure properties similar to native DNA.¹⁴ The synthesized RNA was found to undergo irreversible changes of secondary structure as detected by changes of optical properties, reactivity of functional groups and susceptibility to enzymatic hydrolysis. Prior to this work, only DNA had been shown to exhibit structural properties of this kind. It should be noted that complementary RNA is not synthesized in a highly ordered, DNA-like form. Immediately after synthesis, the RNA appears to be disordered. The formation of ordered RNA structure occurs during the CsCl isolation procedure.

The reaction scheme of Figure 2 summarizes the information obtained from the experiments discussed above. The diagram illustrates how self-complementary strands of RNA might be synthesized and how they could interact to form a DNA-like ordered structure. The RNA strands are shown being assembled on both strands of a single DNA molecule. Although it appears improbable that the polymerization process occurs simultaneously on both strands, we have illustrated the reaction in this fashion simply to demonstrate how complementary ribopoly-

Figure 2. Proposed mechanism for complementary RNA synthesis.

mers are formed at complementary polynucleotide sequences along the DNA template. The formation of an enzyme-RNA-DNA complex is purely speculative.

MESSENGER RNA

Studies in several laboratories, as well as unpublished results by ourselves, have shown that the RNA synthesized by the DNA-directed enzyme stimulates amino acid incorporation into an acid-insoluble material during protein synthesis.^{15,16} Although no specific protein or peptide has been isolated, the results strongly suggest that complementary RNA functions as an intermediary between DNA and protein synthesis. Recently, Champe and Benzer¹⁷ and Yanofsky and Spiegelman¹⁸ have independently arrived at the conclusion that only one of the DNA double strands is used to transcribe information. The synthesis of self-complementary RNA molecules is not at variance with the concept of a single stranded RNA messenger. However, it is important to determine whether the *in vivo* mechanism of the DNA-directed RNA synthesis is consistent with the *in vitro* data which we have obtained.

RNA-DIRECTED SYNTHESIS OF RNA

The specificity of RNA polymerase for a deoxypolynucleotide primer has recently come into question as a result of further studies from our laboratory.¹⁹ We have found that highly purified preparations of this enzyme catalyze ribonucleotide polymerization in the presence of RNA as well as DNA primers. Synthetic ribopolymers such as poly C and poly A, prepared by the action of polynucleotide phosphorylase, are very effective primers. Our results suggest that the ribopolynucleotide directs the polymerization of a complementary RNA strand in a fashion similar to the DNA-directed reaction. Hence, poly C and poly A separately prime the synthesis of poly G and poly U respectively. Careful examination of the relative priming abilities of DNA, poly C and the 1:1 poly A + poly U complex, at different stages of RNA polymerase purification, indicate little change in the ratio of these two activities (Table 1). Studies with purified preparations of RNA polymerase, subjected to electrophoresis at various pH's, indicate that the DNA-

Table 1

RNA SYNTHESIS WITH VARIOUS POLYNUCLEOTIDE PRIMERS: COMPARISON OF ACTIVITIES AT DIFFERENT STAGES OF RNA POLYMERASE PURIFICATION

Specific activity*			Ratio of specific activities	
DNA	Poly C	Poly A + Poly U	DNA/Poly C	DNA/Poly A + Poly U
138 ± 18	113 ± 10	91 ± 4	1.22	1.52
438 ± 38	355 ± 10	260 ± 10	1.23	1.68
660 ± 40	450 ± 20	310 ± 30	1.46	2.12

*Sp. A. = μ moles of NMP³² incorporated/10 min./mg protein at 30°.

and RNA-directed polymerizing activities migrate as a single entity (Figure 3). Although it appears that only one enzyme is involved, the possibility of two enzymes cannot be excluded. DNA is more effective in priming RNA synthesis than the RNA homopolymers, and natural RNA's show little or no activity with this highly purified enzyme; however, relatively crude extracts

Figure 3. LKB electrophoresis of RNA polymerase; 33 hours at 800 V., pH 6,7 in .01 M phosphate buffer

from *M. lysodeikticus* do show significant RNA synthesizing activity in the presence of viral RNA's.¹⁹ The presence or absence of specific RNA-directed polymerizing enzymes in microbial extracts has not been definitely established at this time.

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STRUCTURE-ACTIVITY CORRELATIONS OF ACTINOMYCINS AND THEIR DERIVATIVES*

By

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Actinomycins¹ are highly toxic peptide-containing antibiotics. They are produced by strains of *Streptomyces antibioticus* as mixtures of closely related substances, which may be resolved into their components by countercurrent distribution and partition chromatography.²

Previous work has shown that low concentrations of actinomycin may selectively and completely inhibit RNA biosynthesis in mammalian cells in culture (as well as in some bacteria), while permitting normal growth of the RNA viruses Mengo and poliovirus.³⁻⁵ These observations suggested that virtually the entire complement of the RNA of uninfected cells is produced in a DNA-dependent reaction, and experiments conducted with purified mammalian and bacterial extracts capable of catalyzing such a reaction have demonstrated that it is exceedingly susceptible to inhibition by actinomycin.^{6,7} Moreover, several authors have reported that actinomycin readily complexes to DNA.⁸⁻¹⁰ Since the latter acts as the template for RNA synthesis, the binding of actinomycins to DNA provides a concrete physical basis for its inhibition of RNA biosynthesis.

It has been reported that the antitumour¹¹ and antibiotic² potency of natural actinomycins and their synthetic derivatives may be altered by structural changes affecting different parts of the molecule. These facts made it appear worthwhile to seek a correlation between the inhibition which a compound displayed toward the growth of cells in culture and bacteria, its action on DNA-dependent enzymic RNA synthesis, and its binding to DNA. The results reported in this paper demonstrate that these properties of actinomycins are closely correlated.

RESULTS

Growth inhibition of HeLa cells: When actinomycin is added to the growth medium mammalian cells in culture respond in one of two ways, depending on the concentration of the antibiotic: I. concentrations of actinomycin 2-5 fold greater than those permitting slight residual mitotic activity inhibit cell division immediately, and some loss of cells from the surface of the culture dish may begin within 4 hours; II. at a marginal concentration, some mitotic activity persists for approximately 24 hours after which the cells begin to detach slowly from the surface of the Petri dish. The inhibitory properties of the various actinomycins (Figure 1) have been classified according to the concentration required to produce each response, and the results shown in Table 1, Column A, I and II. It is seen that the requirements for minimal growth inhibition by the compounds listed extend over more than a thousand-fold range of concentrations. Changes involving

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the pentapeptide portions of the molecule (Figure 1) may affect its ability to inhibit the growth of HeLa cells; for example, actinomycin F_1 is reproducibly less active than actinomycins C_1 , C_2 or C_3 , confirming expectations based on previously reported observations in animals. Actinomycin X_2 is perceptibly more active than any of its reduction products. As might have been anticipated from previous results^{2,11} structural changes involving the nuclear amino group yield products whose activity may be much lower than that of the parent compounds.

Figure 1. Structures of some actinomycins according to Brockmann.² The asterisks show the hydroxyl groups which are acylated to yield the corresponding acetates. Derivatives which were used in the present study having structural changes involving the aromatic amino group are as follows: desaminoactinomycin and chloroactinomycin, in which the amino group is replaced by hydroxyl and chlorine respectively; N-substituted actinomycins, in which one hydrogen of the amino is replaced by β -aminoethyl, p -aminophenyl, methyl, β -diethylaminoethyl, and amino groups.

Abbreviations: Meval - N-methyl valine; Sar - sarcosine; Pro - proline; Hypro - hydroxyproline; a-Hypro - allohydroxyproline; Val - valine; Thr - threonine.

The action of the acetates of actinomycins $X_{0\beta}$ and $X_{0\alpha}$ should be noted in relation to that of the parent unacylated compounds. $X_{0\alpha}$ was more active than its acetate in causing immediate and delayed growth inhibition. Likewise, $X_{0\beta}$ was somewhat more effective in causing immediate growth inhibition than $X_{0\beta}$ acetate, but after 24 hours' incubation this difference could no longer be detected. It seems reasonable to suppose that this might be due to slow deacetylation of $X_{0\beta}$ acetate, yielding the parent compound $X_{0\beta}$.

Inhibitory effects of actinomycins on growth of *B. subtilis*: The results of a representative experiment are shown in Table 1, Column B. The order of inhibitory potency resembles that found for HeLa cells, but is not identical with it. It has already been shown^{2,20} that the actinomycin derivatives which we have found to be ineffective in inhibiting the growth of HeLa cells are inactive against *B. subtilis*, and the data in Table 1 confirm this. These results differ some-

Table 1

Actinomycin	A HeLa		B <i>B. subtilis</i>	C <i>E. coli</i> RNA-polymerase			D HeLa RNA-polymerase			E Binding to DNA
	I $\mu\text{g/ml}$	II $\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	m μ moles incorporated	% Inhibition	$\mu\text{g/ml}$	m μ moles incorporated	% Inhibition	
X ₂	0.01 (1)	0.0025 (1)	0.1 (1)	0.5	0.785	59.0 (1)	5	0.114	36.3 (2)	(1)
C ₃	0.02 (2)	0.005 (2)	0.25 (2)	0.5	0.93	52.5 (2)	5	0.115	35.7 (2)	0
C ₁	0.02 (2)	0.005 (2)	0.25 (2)	0.5	0.98	49.0 (3)	5	0.115	35.7 (2)	(2)
C ₂	0.02 (2)	0.005 (2)	0.25 (2)	0.5	0.97	50.5 (3)	5	0.109	39.0 (1)	0
X _{0a}	0.02 (2)	0.01 (5)	0.35 (5)	0.5	1.02	46.8 (5)	5	0.132	26.2 (8)	(2)
X _{0a} acetate	0.05 (6)	0.02 (6)	0.5 (6)	0.5	1.11	42.2 (6)	5	0.115	35.7 (2)	(2)
F ₁	0.2 (7)	0.10 (7)	1.0 (7)	0.5	1.19	37.9 (8)	5	0.123	31.3 (6)	(2)
X _{0b}	0.5 (8)	0.10 (7)	1.5 (9)	0.5	1.16	39.6 (7)	5	0.122	31.4 (6)	(2)
X _{0b} acetate	1.0 (9)	0.10 (7)	2.5 (10)	0.5	1.24	35.4 (9)	5	0.128	28.5 (8)	(8)
N-Methyl C ₂	1.0 (9)	0.10 (7)	1.0 (7)	1.0	1.39	27.1 (10)	25	0.07	60.9 (10)	(7)
N- β -Diethylaminoethyl C ₃	1.0 (9)	0.10 (7)	2.5 (10)	1.0	1.63	15.1 (11)	25	0.124	30.7 (11)	(9)
Desamino C ₂	>10 (i)	10 (i)	>10 (i)	10	1.93	-- (i)	25	0.181	--	(i)
Chloro C ₃	>10 (i)	0.1 (†)	>10 (i)	10	1.89	1.5 (i)	25	0.102	43	(i)
N- β -Aminoethyl C ₃	>10 (i)	1.0 (†)	>10~ (i)	10	1.81	5.7 (i)	25	0.181	--	(i)*
N-p-Aminophenyl C ₃	>10 (i)	10 (i)	>10 (i)	10	1.90	1.1 (i)	25	0.194	--	(i)
N-Amino C ₃	>10 (i)	1.0 (†)	>10 (i)	10	1.83	4.7 (i)	25	0.085	52.5	(i)
Control					1.92	--		0.180	--	

The numbers in parentheses represent the ranking, in order of decreasing inhibitory effectiveness, of the compounds tested in each experiment.

(i) considered inactive
0 compound not tested

> indicates that the highest concentration tested did not inhibit
† observed activity considered due to transformation to active compound - see text

Column A - Concentration of compound required to inhibit division of HeLa cells (I) without delay (II) after 24 hours incubation. Aliquots of HeLa (Strain S-3) spinner cell cultures were added to plastic Petri dishes at 2.5×10^4 per dish. Cell counts were performed after 8 and 24 hours on representative areas delineated on each plate, as previously described.¹² The medium was changed immediately prior to the second cell count, appropriate concentrations of aqueous solutions of the test compounds added and cell counts repeated at 24-hour intervals. Two plates were used for determining the activity of each concentration of every compound.

Column B - Concentration of compound required to inhibit completely the growth of *Bacillus subtilis* (strain Marburg, wild type). Equal aliquots of an exponentially growing culture of the bacterium were inoculated into large test tubes containing warmed nutrient broth and various concentrations of the test compounds. The optical density of the suspension was recorded before and after one hour's incubation at 37° with rapid rotary shaking. The concentrations listed are the minimum levels of each compound required to prevent any increase in optical density (600 m μ) during the 60-minute incubation period.

Columns C and D - Effect of actinomycins and derivatives on *E. coli* and HeLa RNA-polymerases respectively. Experimental conditions as described for Figure 2.

Column E - Ranking of actinomycins and derivatives with respect to their affinity for binding to DNA, measured as described for Figure 4. * Interactions observed between compound and DNA, but not of type characteristic of biologically active forms. See spectra in Figure 3.

what from those reported by Brockmann and collaborators^{2,20} for the relative activities of $X_{O\beta}$, $X_{O\alpha}$, and their acetates. They observed a ten-fold greater activity with $X_{O\beta}$ than with $X_{O\beta}$ acetate, and a five-fold greater activity with $X_{O\alpha}$ than with $X_{O\beta}$. Our findings resemble qualitatively those of the above authors, and the relatively small quantitative discrepancies may be attributable to slight differences in culture conditions, bacterial strain, or experimental design.

Effects of actinomycins on *E. coli* and HeLa RNA-polymerase: *E. coli* RNA polymerase is strongly inhibited by actinomycin. The data in Figure 2 demonstrate the inhibition of incorporation of UTP³² as a function of actinomycin concentration. A comparison of the effects of various actinomycins and their derivatives is given in Table 1, Column C.

In this test the various actinomycins and their derivatives show a graded series of activities which resembles that found for toxicity to HeLa cells and *B. subtilis*. The inhibition of *E. coli* RNA-polymerase by actinomycin is not due to an effect on the enzyme directly, but results from the binding of the antibiotic to the DNA primer. This is shown by the fact that the inhibition due to a given concentration of actinomycin is not affected by increasing the concentration of enzyme, but may be diminished to any desired extent by suitable additions of DNA. Similar observations have been recorded⁶ for HeLa RNA-polymerase. For those actinomycins whose structural differences involve only the peptide portions of the molecule, the resulting variations in effectiveness of enzyme inhibition can best be demonstrated when the concentration of DNA in the reaction mixture is not far above the saturating level. This may be seen by contrasting the findings summarized in Table 1, Column D with those in Column C. In this experiment the various compounds were assayed for inhibitory potency using an enzyme preparation, obtained from HeLa cell nuclei, which is not dependent on added DNA for ribopolynucleotide synthesis. The active fraction is very viscous since it contains large amounts of DNA, thereby reducing the precision of the assay, and correspondingly higher concentrations of actinomycins are required to obtain degrees of inhibition comparable to those observed with the bacterial enzyme preparation (Figure 2).

It is of interest that 3-chloroactinomycin and N-amino actinomycin, which were inert in the reaction catalyzed by the bacterial enzyme preparation, show appreciable inhibitory activity with the HeLa enzyme. It should be noted that some of the N-substituted actinomycins are somewhat unstable, reverting spontaneously to the parent compounds. Furthermore, chloroactinomycin is notably reactive, and readily yields free or N-alkyl actinomycins on treatment with ammonia or alkylamines.² Since the HeLa enzyme, in contrast to the bacterial enzyme, is best assayed in the presence of high concentrations of ammonium sulphate, the varying behaviour of these otherwise inactive actinomycin derivatives in the two enzyme systems is probably due to their partial transformation to active forms in the HeLa incubation mixture.

Effect of actinomycins on pseudouridine incorporation into polynucleotides: Pseudouridine triphosphate (Ψ UTP) has been shown to be incorporated into ribopolynucleotides by RNA polymerase preparations from mammalian¹⁶ and bacterial^{17,18} sources. The incorporation pattern of this rare nucleotide differs from that of uridine nucleotide^{16,17} in the presence of uridine triphosphate. Nevertheless, the degree of inhibition of incorporation of radioactive Ψ UTP by the complete series of compounds listed in Table 1 paralleled that found for UTP.

Effect of DNA on spectral properties of actinomycins and derivatives: Kirk⁸ found that a biologically active actinomycin complexes with DNA whereas actinomycinic acid, which is formed on hydrolysis of the lactone rings and which is biologically inactive, does not. The

Figure 2. (a) Inhibition of HeLa RNA-polymerase by actinomycin. The enzyme was prepared and assayed, using ³²P-labelled uridine triphosphate (UT³²P) to measure incorporation of uridylic acid into RNA.¹⁵ The reaction mixture (final volume 0.5 ml) contained 125 μg of DNA in the form of the "aggregate" enzyme.¹⁵

(b) Inhibition by actinomycin of RNA-polymerase from *E. coli* B. The enzyme was prepared and enzymatic reactions performed according to Chamberlin and Berg.¹³ The enzyme fraction used was totally dependent for activity on added DNA and nucleoside triphosphates. UT³²P, prepared as previously described,¹⁴ was used as tracer for nucleotide incorporation into acid-insoluble form. The reaction mixture, which contained 18.5 μg of enzyme protein, was incubated for ten minutes at 37°. The reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 5 per cent in the presence of carrier yeast RNA and serum albumin. The resulting precipitate was washed three times with TCA, dissolved in ammonium hydroxide, plated, and its radioactivity measured in a thin-window gas flow counter. A single preparation of calf thymus DNA (Sigma Chemical Co., St. Louis, Missouri) was used for all studies with the enzyme from *E. coli*. The concentration of DNA-P was 176 mμ moles/ml, a level which was just above that saturating the enzyme under our conditions.

changes seen in the presence of DNA resemble those which may occur on alkylation of the amino group, namely, broadening of the absorption peak and its shift to longer wavelengths. We have examined the spectral changes of the actinomycins and their derivatives which are observed on interaction with DNA. The results are summarized in Figure 3, where the difference spectra in the visible region are shown. Under identical conditions and concentrations the spectral changes observed in the presence of DNA were very similar in position and magnitude for actinomycins C_1 , C_2 , C_3 , X_0 , $X_0\alpha$ acetate, F_1 and $X_0\beta$, but differed quantitatively from those observed for $X_0\beta$ acetate.

N-methyl and N- β -diethylaminoethyl actinomycins were found to be active in toxicity and enzymatic tests. While these compounds interacted with DNA in solution, and their spectral behaviour qualitatively resembled that of the parent substances, the optical changes were smaller, on a molar basis, than those found for the parent compounds. The contour of the difference spectrum seen with N-methyl actinomycin is similar to that of the parent substance, but the peaks and crossover points occur at somewhat different wavelengths. For this reason it appears unlikely that the spectral changes, and therefore the binding of N-methyl actinomycin to DNA, are the result of a substantial degree of contamination of the preparation with free actinomycin. It would follow that methylation of the amino group does not destroy the biological activity of the actinomycin. N-diethylaminoethyl actinomycin possesses measurable, but considerably less, biological activity than its parent compound. If the spectral changes induced by DNA were assumed to be due entirely to the presence of unsubstituted actinomycin, the concentration of the latter would be of the proper order of magnitude to account for the observed activity, provided the N-substituted species were totally inactive. Desaminoactinomycin, 3-chloroactinomycin and N-p-aminophenyl actinomycin were inert, or showed optical changes which were minor in degree and differed qualitatively from those of parent compounds. B- β -aminoethyl actinomycin, which did not inhibit the growth of HeLa cells or *B. subtilis*, unexpectedly showed marked spectral changes in the presence of DNA; as is shown in Figure 3, these differed from those observed for the biologically active compounds. This suggests that while actinomycins and their derivatives may interact with DNA in various ways, probably only one type of interaction is effective in suppressing DNA-dependent RNA synthesis.

The effect of varying concentration of DNA and actinomycin on the spectral changes associated with complex formation deserves comment. For a given level of actinomycin C_1 increasing DNA concentrations induce progressive and approximately proportional changes in spectral behaviour (cf. Figure 4 and ref. 10), until a level is reached beyond which additional DNA has no further effect. Raising the actinomycin concentration at this level of DNA leads to further complex formation, as shown by the accompanying increased spectral changes. Such dependence of binding on actinomycin concentration might be observed if all available actinomycin were bound and the resultant complex almost entirely undissociated, at DNA concentrations whose effect is maximal for a given actinomycin level. This interpretation is supported by the fact that the absolute value of the spectral changes observed at a fixed level of DNA is exactly proportional, over a wide range of actinomycin concentrations, to the amount of actinomycin present. This would not be observed if, at equilibrium, more than a few per cent of the actinomycin remained uncomplexed to DNA.

The binding of a series of actinomycins has been examined at different DNA levels. Some of the findings are shown in Figure 4. A number of the actinomycins tested (C_1 , X_2 , $X_0\alpha$, $X_0\alpha$

Figure 3. Difference spectra obtained by recording (Cary spectrophotometer Model 11) the spectrum of a solution containing an actinomycin and DNA against a reference solution containing only the same concentration of the actinomycin. All solutions were 0.01 M NaCl - 0.01 M Tris buffer (pH 7.4). The spectra of actinomycins C₁, C₂, C₃, $\bar{X}_2$, $X_{0\alpha}$, $X_{0\alpha}$ acetate, $X_{0\beta}$, $X_{0\beta}$ acetate, F₁, N-methyl C₂ were obtained at a concentration of 8×10^{-6} M in the presence of 176 μ moles/ml of calf thymus DNA- $\bar{P}$. The spectra of the remaining derivatives were recorded at 10^{-5} M in the presence of 440 μ moles/ml of the same calf thymus DNA preparation. Although the changes induced in solutions of actinomycins by DNA affect all portions of the actinomycin spectrum, we have examined only the visible region 350-600 μ .

acetate, $X_{O\beta}$, $X_{O\beta}$ acetate, F_1) differ structurally in ways which might be expected to influence the stereochemical properties of their complex with DNA. Therefore it cannot be assumed that the molar extinction coefficients of the various actinomycin-DNA complexes are identical, and since these have not been established, for each compound, the results have been charted as shown. The maximal optical density change observed, and by inference the maximal binding at a

Figure 4. Binding of actinomycins to DNA was measured by the decrease in absorption at 425 mμ on addition of standard amounts of DNA. All solutions were 0.01 M NaCl - 0.01 M Tris buffer (pH 7.4). The calf thymus DNA preparation contained 134.5 mμ moles of phosphorus per unit optical density (260 mμ) and a hyperchromic effect of 42.5 per cent (pH 7.0, 260 mμ).

given concentration of the antibiotic, occurs at a lower concentration of DNA for actinomycin X_2 than for C_1 or $X_{O\beta}$ acetate. Curves of this type have been recorded under identical conditions also for actinomycins F_1 , $X_{O\alpha}$, $X_{O\alpha}$ acetate, and although the biological activity of these compounds differs significantly from that of C_1 , their spectral behaviour at different DNA concentrations is indistinguishable from the curve shown for C_1 . The simplest explanation of these findings is provided by the assumption that the differences in optical behaviour (and therefore presumably in binding) between the various actinomycins reflect the degree of dissociation of the particular actinomycin-DNA complexes. Actinomycin X_2 , for example, would be assumed to reach a binding equilibrium, characteristic for any given drug concentration, at a lower concentration of DNA. It is possible that the binding differences may reflect differences in the numbers and/or properties of sites available on DNA for the various actinomycins.

These experiments have been conducted with a single DNA preparation at a single pH. Variations of concentration of NaCl over the range 0-1.0 M did not affect the spectral changes produced by a given concentration of DNA, and the effect of changing other variables (e.g., polyvalent cations or anions, temperature, or different DNA preparations) have not been systematically explored. Heating the DNA preparation (100°, 10 minutes, 0.01 M NaCl, 0.01 M tris buffer pH 7.4) followed by rapid cooling, reduced its ability to bind a given amount of actinomycin by approximately 15 per cent.

DISCUSSION

In Table 1 the various compounds used in this study have been ranked in decreasing order of potency for each test system in which they have been examined. Except for the HeLa enzyme assay, the relative activities, with minor deviations, correlated in all tests. In the case of HeLa RNA-polymerase it is felt that the viscosity and high DNA content of the enzyme preparation are probably responsible for imprecisions of sufficient magnitude to blur any differences in activity of those actinomycins whose structural dissimilarities are restricted to the composition of the peptide chains. However, even the HeLa enzyme assay could easily distinguish between the N-substituted and parent compounds.

The differences in tumor-inhibitory activity reported for actinomycins *in vivo*¹¹ closely parallel those recorded above for the compounds used in both studies. Although the various systems available for estimating the potency of actinomycins possess approximately equivalent resolving power for detecting activity differences, it is apparent that the most sensitive measure is provided by the growth response of the HeLa cell line. The precision of this procedure is at least equal, if not superior, to that of the other available test systems.

It may be recalled that actinomycin does not affect the growth of several RNA viruses in cultured mammalian cell lines,⁵ and thus appears not to affect the many complex cellular enzymatic and structural properties which are required to sustain virus growth. Therefore it is noteworthy that the activities of the large family of compounds examined in this study appear, with the exceptions noted above, to correlate strongly at the level of the intact animal, the intact mammalian and bacterial cell, the bacterial enzyme assay, and, to a considerable extent, even with the optical changes produced on complexing with DNA. These facts make it highly probable that the toxicity and biological activity of actinomycin are due exclusively to its binding to DNA and to the consequent interference with DNA metabolism, the molecule being otherwise innocuous to other physiological processes. This is a further illustration of the remarkable specificity of action characteristic of many antibiotics.

No explanation is available for the fact that several actinomycins having lower biological activity appear to bind to DNA as efficiently as C₁. Demonstration of small variations in binding might require ionic conditions other than those used in these experiments. Alternatively, differences in biological activity may reflect not merely the quantitative aspects of binding of actinomycins, but might also be partially determined by the structural features of the different bound forms or of the complex with DNA.

While structural features of actinomycins affect their reaction with DNA, the base composition of the latter influences the extent of binding and the degree of inhibition of enzymatic RNA synthesis. Synthetic deoxyadenylichthymidylic copolymer²¹ (dAT) directs the synthesis by RNA-polymerase, of a corresponding polyriboadenylic-uridylic polymer²² in a reaction which does not require the presence of guanosine or cytidine triphosphates. This synthesis is totally resistant to very elevated concentrations of actinomycin (150 µg/ml).²³ In contrast, when the naturally-occurring dAT polymer²⁴ which is found in crab DNA is used to prime RNA formation, all four ribonucleoside triphosphates are required for optimal synthesis, the RNA produced contains approximately 2.5 per cent guanine and cytosine nucleotides,²³ and the reaction is reproducibly inhibited (25 per cent) by actinomycin (100 µg/ml). Moreover, crab dAT induces small but definite spectral shifts, resembling those normally produced by DNA in actinomycin solutions, whereas synthetic dAT does not.²³ In tests of a series of DNA preparations from different sources the

amount of actinomycin bound, and the degree of inhibition of RNA-polymerase reactions were found to be a function of the guanine-cytosine content of the DNA.²³ The results of these and other experiments which will be described more fully²³ demonstrate that actinomycin binding to DNA and its inhibition of RNA synthesis is mediated through the guanine residues of the primer template. This fact fulfills the prediction of Kersten²⁴ who found that of the normal constituents of nucleic acids only deoxyguanosine could induce spectral changes of actinomycins similar to those produced by DNA.

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BASIS OF ACTINOMYCIN ACTION*

By

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Actinomycin inhibits DNA-dependent RNA synthesis of intact cells¹ and enzymes from mammalian² and bacterial³ sources, but not the growth of RNA-viruses in animal cells.^{1,4} The inhibition of RNA synthesis appears to account completely for the antibiotic and cytotoxic action of actinomycin and is attributable to the fact that the antibiotic is tightly bound^{5,6} to DNA.

Structural changes in the actinomycin molecule which affect its biological activity also influence, in a parallel way, its binding to DNA and its inhibition of the RNA polymerase reaction.³ The experiments described below suggest a basis for the effect of actinomycin in that the base composition of DNA primers may influence the extent of actinomycin binding and thereby the degree of inhibition of the different reactions catalyzed by RNA-polymerase. The binding of actinomycin appears to be absolutely dependent on the presence of guanine residues in the DNA primers. Kersten⁷ showed that high concentrations of deoxyguanosine and several other guanine and adenine compounds could alter the spectral properties of actinomycin solutions. Our data show that only guanine residues in DNA are indispensable for actinomycin action.

Recently Hurwitz *et al.* have reported on the effect of actinomycin on some aspects of bacterial RNA-polymerase activity.⁸ Several of their findings differ from those recorded below.

MATERIALS AND METHODS

The standard assay conditions used in the experiments, and the isolation of RNA-polymerase from *E. coli B* were as described by Chamberlin and Berg,⁹ and the incubations were carried out for 15 min at 37° unless otherwise noted.

Incorporation of precursors into RNA under standard conditions in the absence of DNA was less than 0.02 μ moles/15 min for 4 NTP,^{**} and smaller for single NTP; these control values were always subtracted from the results recorded below. All reactions contained 4.1 μ g enzyme protein/0.25 ml. Synthesis of acid-insoluble polyribonucleotides was measured by the incorporation of one of the following precursors: C¹⁴-ATP, obtained from Schwarz BioResearch; UTP³², Ψ UTP³², CTP³², GTP³², synthesized as previously described,¹⁰ in which the proximal phosphate was radioactive. Incorporation of radioactive pyrophosphate (PP³²) into the terminal pyro-

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** Abbreviations: NTP-ribonucleoside triphosphate; ATP, CTP, GTP, UTP; AMP, CMP, GMP, UMP; poly A, poly C, poly G, poly U-5'-triphosphates, 3'(2') monophosphate, 3'-5' polymeric monophosphates respectively of adenosine, cytidine, guanosine, uridine. TTP - thymidine triphosphate. DNA - deoxyribonucleic acid; RNA - ribonucleic acid.

phosphate position of nucleoside triphosphates ("exchange reaction") was determined as follows: PP^{32} , prepared according to Jones *et al.*,¹¹ was incubated with the components of the standard assay mixture, in the absence of Mn^{++} , at a concentration of 1.6 μ moles/ml; $MgCl_2$ was present at 8 μ moles/ml. The reaction was stopped by chilling, adding a large excess of non-radioactive phosphate and pyrophosphate buffers, carrier bovine serum albumin and yeast RNA (2 mg each per reaction vessel) and trichloroacetic acid (final conc. 5 per cent). After centrifugation, the supernatant solution was treated with charcoal; the latter was washed four times with acetate buffer (0.1 M pH 4.5) containing phosphate and pyrophosphate (0.03 M), transferred to a planchet, dried, and the adsorbed radioactivity measured in a thin-window gas-flow counter. Nucleic acid concentrations were based on the phosphorus content determined according to Fiske and SubbaRow.¹² Protein was measured according to Lowry *et al.*¹³ Actinomycin concentrations were based on the absorption of a standard solution of 25 μ g/ml giving an O.D. (440 m μ) of 0.358 in 0.01 M Tris pH 7.4 with 1 cm light path. The absorption of pure actinomycin is appreciably greater than this value, which was established several years ago for a preparation containing inert material.

DNA Preparations: All DNA solutions were made in 0.01 M Tris (pH 7.4) -0.01 M NaCl, except for crab dAT, which was dissolved in 0.75 M NaCl -0.05 M phosphate (pH 6.7). Highly polymerized calf thymus DNA was purchased from Sigma Chemical Co., St. Louis, Mo.; DNA from *Micrococcus lysodeikticus* and *Tetrahymena pyriformis* was prepared by the method of Marmur,¹⁴ according to a modification suggested by Dr. N. Sueoka. Calf thymus apyrimidinic DNA was prepared according to Habermann,¹⁵ and calf thymus apurinic DNA according to Tamm *et al.*¹⁶ Synthetic deoxyadenylic-deoxythymidylic (dAT) and deoxyguanylic-deoxycytidylic polymers (dGC), crab dAT,¹⁷ a preparation of calf thymus apyrimidinic DNA, and DNA from bacteriophage ϕ X-174 were generously provided by Drs. A. Kornberg, N. Sueoka, V. Habermann and R. L. Sinsheimer respectively. Non-radioactive NTP was obtained from Pabst Laboratories and Sigma Chemical Co. We thank Dr. S. B. Weiss for a gift of GTP³².

RESULTS

Binding of actinomycin to DNAs of varying base composition: Guanine residues are indispensable for actinomycin binding to DNA. The data in Figure 1 show that synthetic dAT copolymer and apurinic acid do not bind actinomycin. However, the naturally-occurring dAT copolymer of crab testis, which contains approximately 2.5 per cent GC,¹⁸ does cause slight optical changes in actinomycin solutions, which qualitatively resemble those normally produced by native DNA. Since apyrimidinic DNA and dGC polymer both complex actinomycin, binding requires only the guanine residue of the GC base pair.

The base composition of DNA may affect its binding of actinomycin as shown in Figure 2. *M. lysodeikticus* DNA (G content 37 per cent)¹⁷ binds actinomycin much more effectively than does *T. pyriformis* DNA (G content 12.5 per cent),¹⁹ calf thymus DNA (G content 22 per cent)¹⁷ being intermediate. All three DNA preparations were equally "native" as judged by "hyperchromic effect" following acid hydrolysis or alkali treatment. Binding by nucleic acid is influenced also by the intactness of its helical structure as shown by two facts: (a) heating the DNA (100°, 10 min), followed by rapid cooling, significantly decreases the amount of actinomycin bound; (b) despite its higher G content (24 per cent) single-stranded²⁰ ϕ X-174 DNA binds actinomycin no more effectively than *T. pyriformis* DNA.

Figure 1. Difference spectra, obtained in Cary Spectrophotometer Model 11, of actinomycin solutions (O.D. 440 $m\mu$ = 0.78, 1 cm light path) read against the same solutions containing the DNA preparations shown. Note the differences in scale of the ordinates. The observed differences for synthetic and crab dAT were obtained under similar conditions but with a 5 cm light path. The concentrations of DNA-P were: calf thymus 108 $m\mu$ moles/ml; aprimidinic 1.5 μ moles/ml; synthetic dAT 1.65 O.D. units (260 $m\mu$)/ml, estimated 276 $m\mu$ moles/ml; crab dAT 0.429 O.D. units (260 $m\mu$)/ml, estimated 71.6 $m\mu$ moles/ml; dGC 158 $m\mu$ moles/ml; apurinic 1.61 μ moles/ml.

Figure 2. Actinomycin binding by DNA of different base composition, measured by change in absorbance at 425 m μ (initial O.D. 425 m μ without DNA = 0.635). All solutions 0.01 M NaCl - 0.01 M Tris (pH 7.45). x—x *M. lysodeikticus* DNA; (x) - heated *M. lysodeikticus* DNA; ●—● calf thymus DNA; (●) - heated calf thymus DNA; o—o *T. pyriformis* DNA; ϕ -DNA from bacteriophage ϕ X-174.

Inhibition of polyribonucleotide synthesis by actinomycin: (a) Heteropolymer formation.—

The extent of incorporation into RNA of a given NTP by RNA-polymerase reflects the base composition of the DNA primer. For example, DNA from *M. lysodeikticus* primes the incorporation of CTP more efficiently than does calf thymus DNA. As shown in Figure 3, the incorporation of both UTP and CTP into RNA is more sensitive to actinomycin when the reaction is primed with *M. lysodeikticus* DNA than with DNA from calf thymus or *T. pyriformis*. This holds throughout a wide range of actinomycin concentrations and also (*vide infra*) for the NTP-PP exchange reaction. This finding is noteworthy since: (a) at the concentrations of DNA used, its capacity for binding actinomycin is not saturated below antibiotic levels of approximately 9 μ g/ml; (b) on the basis of arguments presented elsewhere³ it is unlikely that more than a very small fraction of actinomycin is not bound in the presence of adequate concentrations of native DNA. The equal amounts of the three types of DNA employed in this experiment, corresponding to positions of identical slope on the respective velocity/DNA concentration curves, must accordingly be presumed to have bound the same quantity of actinomycin. Therefore, it is of interest that the antibiotic impairs the priming action of *M. lysodeikticus* DNA for RNA synthesis to a significantly and reproducibly greater extent than that of calf thymus or *T. pyriformis* DNA. This observation might result if the binding sites for actinomycin, of which guanine is in some way an indispensable component, were evenly distributed throughout *M. lysodeikticus* DNA so that no regions of appreciable length are spared the inhibitory action of the drug. DNA from calf thymus and *T. pyriformis* could then be expected to be more heterogeneous with respect to structural require-

Figure 3. Actinomycin C₁ inhibition of incorporation of CMP³² (A) and UMP³² (B) into RNA. Standard conditions, 196 mμ moles DNA-P/ml of each preparation. 100 per cent incorporation with CTP³² (sp. act. 9.1×10^5 cpm/μmole) was 2.99, 2.36, and 2.13 mμ moles, and with UTP³² (sp. act. 4.2×10^6 cpm/μmole) 3.10, 0.67, and 4.09 mμ moles for calf thymus (○—○), *M. lysodeikticus* (●—●) and *T. pyriformis* (x—x) DNA respectively.

ments for actinomycin binding and consequent inhibition of RNA synthesis. This expectation may be related to the capacity of these DNA preparations to prime the synthesis of poly A and poly U.

(b) Effect of actinomycin on dAT-primed RNA synthesis.—Synthetic dAT copolymer consists of perfectly alternating deoxyadenylic and thymidylic acids,²¹ and when used to prime RNA synthesis the product contains alternating adenylic and uridylic residues.²² The data in Table 1 show that this RNA synthesis is totally resistant to actinomycin, reflecting the absence of actinomycin binding to the primer (Figure 1). The lack of effect of actinomycin on dAT-primed RNA synthesis is in contrast to the action of proflavine, which can strongly inhibit the same reaction (50 per cent inhibition at 1.5×10^{-5} M proflavine).

The light component of *Cancer borealis* testis DNA contains predominantly adenine and thymine.¹⁸ In an experiment in which this DNA was used to prime the DNA-polymerase reaction, dGTP and dCTP were required for completion of the synthetic reaction, the product contained approximately 2.6 per cent GC, and the GC components were scattered through an otherwise almost perfectly alternating sequence of deoxyadenylic and thymidylic acids.¹⁸ We have made similar observations in using this substance to prime RNA synthesis. The omission of GTP and CTP, which does not affect the reaction primed by synthetic dAT, markedly reduced the RNA synthesis directed by crab dAT.*

* This inhibition could be overcome by doubling the concentration of ATP and UTP³² normally used with enzyme assay. However, on electrophoresis of the alkaline hydrolysate of the RNA produced in this manner, radioactivity was detectable in the areas corresponding to GMP and CMP, showing that the commercially available NTP contained impurities.

When UMP³² was incorporated into RNA in the presence of crab dAT, and the product hydrolyzed in alkali, the distribution of radioactivity in the 2'(3') mononucleotides isolated following paper electrophoresis (95.2, 2.9, 1.7, 0.3 per cent in A, U, G, C, respectively) closely resembled that found for DNA replication with TTP³² as radioactive precursor.¹⁸ In addition, the

Table 1

EFFECT OF ACTINOMYCIN C₁ ON RNA SYNTHESIS PRIMED
BY SYNTHETIC AND CRAB "dAT"

Primer	Actinomycin C ₁ (μ g/ml)	m μ moles UMP ³² incorporated	% inhibition
Synthetic dAT	--	2.15	--
Synthetic dAT	100	2.14	0.5
Crab dAT	--	1.98	--
Crab dAT	100	1.50	24.3

The reaction mixture contained 0.005 O.D. units (260 m μ) of the relevant primer in a final volume of 0.25 ml UTP³² (1.16×10^6 cpm/ μ mole) was the radioactive precursor. All four NTP were present at 400 m μ moles/ml each throughout to maintain identical reaction conditions for both primers. Each vessel also contained NaCl 33.6 μ moles/ml and potassium phosphate buffer pH 6.7, 2.4 μ moles/ml 15', 37°.

small amount of GC present in crab dAT suffices to render its priming of RNA synthesis partially, but significantly, susceptible to actinomycin, in contrast to the absolute resistance of the reaction primed by synthetic dAT (Table 1). Actinomycin lowers the rate of synthesis but does not alter the base composition of the RNA produced with crab dAT as primer. The low frequency of guanine residues in crab dAT implies the existence of long guanine-free sequences incapable of binding and therefore insensitive to inhibition by actinomycin. For this reason, it was expected that the RNA produced in the actinomycin-inhibited reaction might contain a lower than normal proportion of C and G, but this was found not to be the case.

(c) Effect of actinomycin on dGC-primed RNA synthesis.—Synthetic dGC consists of a mixture of homopolymers of deoxyguanylic and deoxycytidylic acids,²³ and primes the synthesis, by RNA-polymerase, of polyribonucleotides containing only G and C.⁹ We have found that the incorporation of GMP³² and CMP³² into RNA synthesized in the presence of dGC proceeds independently (Table 2), in contrast to RNA synthesized with dAT as primer. That is, dGC can prime the synthesis of RNA homopolymers whereas dAT, whose nucleotide sequence is an alternating one, cannot. It follows that these homopolymer syntheses are associated with the presence of long repeating sequences in the primer. dGC-primed synthesis of both homopolymeric polyribonucleotides may be inhibited by actinomycin (Figure 4) although the concentration required is greater than that required for comparable inhibition of heteropolymer reactions primed by DNAs containing all four bases.

(d) Effect of actinomycin on homopolymer synthesis primed by various DNA preparations.—Chamberlin and Berg reported⁹ that RNA-polymerase is capable of catalyzing the synthesis of homopolymeric ribopolynucleotides when certain DNA preparations (high in AT) are used to prime the reaction. The data in Figure 4 show the effect of actinomycin on the calf thymus DNA-primed synthesis of polyadenylic, polyuridylic and polypseudouridylic acids. The formation of

Table 2

POLY C AND POLY G SYNTHESIS PRIMED
BY dGC AND *M. lysodeikticus* DNA

Primer	Precursors	Mμ moles incorporated
dGC	GTP ³²	1.07
dGC	GTP ³² + CTP	0.99
dGC	CTP ³²	0.16
dGC	CTP ³² + GTP	0.18
<i>M. lysodeikticus</i> DNA	CTP ³²	0.44
<i>M. lysodeikticus</i> DNA	CTP ³² + ATP + GTP + UTP	3.52

Standard reaction conditions, incubation time 30', 8.2 μg enzyme protein/ml. 2.8 mμ moles of dGC and 49 mμ moles *M. lysodeikticus* DNA were used. The specific activity of CTP³² was 2.61×10^6 cpm/μmole and of GTP³² 2.38×10^6 cpm/μmole. With dGC, CTP³² and GTP³² were present at 200 mμ moles/0.25 ml when used alone; otherwise all NTP's were present at 100 mμ moles/0.25 ml.

Figure 4. Effect of actinomycin C₁ on homopolyribonucleotide synthesis, primer DNA preparation indicated in parentheses in the figure. The reactions primed with dGC contained 400 mμ moles/ml of each of the four NTP; this addition does not affect the nature of the product, but increases the reaction rate somewhat. All other reactions contained 400 mμ moles/ml of the single relevant NTP under standard conditions. 100 per cent incorporation was as follows: poly C (dGC), 0.10 mμ moles; poly G (dGC) 0.80 mμ moles; poly C* (*M. lysodeikticus*) 0.44 mμ moles; poly A 0.74 mμ moles; poly U 0.0196 mμ moles; poly ψU 0.138 mμ moles.

* We have found that *M. lysodeikticus* DNA can prime the formation of poly C (but not poly G, poly A or poly U) whereas calf thymus DNA cannot. This homopolymer synthesis is more

homopolymers is substantially more resistant to actinomycin than that of heteropolymers. Very high concentrations of actinomycin do significantly inhibit homopolymer synthesis in contrast to a previous report.⁸ This inhibition must be specific for a biologically active actinomycin since, (a) equivalent concentrations of biologically inactive derivatives (desaminoactinomycin and N-p-aminophenylactinomycin) which do not bind to DNA,^{3,24} and which do not affect heteropolymer synthesis are totally innocuous to homopolymer formation and (b) a biologically active actinomycin which interferes with homopolymer synthesis does so by binding to the DNA primer, and not by non-specific inhibition of the enzyme (viz., synthetic dAT).

(e) Effect of actinomycin on RNA synthesis primed by ϕ X-174 DNA.—Heated DNA, a portion of which has lost its helical configuration, and ϕ X-174 DNA, known to be single-stranded,²⁰ bind actinomycin less effectively than double-stranded DNA of identical base composition (Figure 2). RNA-polymerase is saturated by a low level of ϕ X-DNA (12 m μ moles DNA-P/ml) as has been shown for heated DNA.²⁵ Below this level, the velocity/DNA concentration curves for DNAs from ϕ X-174 and calf thymus are identical and permit a comparison of their actinomycin-susceptibility. The actinomycin-sensitivity of the reaction primed with ϕ X-DNA is approximately half that found for calf thymus DNA (Figure 5).

Figure 5. Comparison of reaction velocity at varying DNA concentrations, and sensitivity to actinomycin of calf thymus (●—●), and ϕ X-174 DNA (x—x). Standard conditions. Actinomycin C₁ at 0.2 μ g/ml. Calf thymus with actinomycin (o—o). ϕ X with actinomycin (▲—▲). UTP³² was the radioactive precursor.

susceptible to actinomycin than those primed by calf thymus DNA and resembles the reactions primed by dGC. The poly C was characterized as follows: paper electrophoresis of an alkaline hydrolysate of the product showed 97.5 per cent of the radioactivity associated with the CMP region, 2.5 per cent with the UMP region (probably the result of deamination occurring during hydrolysis). When the CMP region was eluted and chromatographed on paper in a borate buffer system all the radioactivity moved as 2'(3') CMP.

The concentration of ϕ X-DNA required to saturate the synthetic reaction is considerably higher than for the exchange reaction.

(f) Effect of actinomycin on NTP-PP³² exchange reaction.—RNA-polymerase catalyses a DNA-dependent exchange reaction between a single NTP and inorganic PP. This reaction is less sensitive to actinomycin than the synthetic reaction with the same DNA preparations (Figure 6). Nevertheless, the difference in actinomycin-sensitivity between synthetic reactions primed by

Figure 6. Actinomycin inhibition of NTP-PP exchange reaction. With crab dAT, 400 $m\mu$ moles each of ATP and UTP were present, otherwise each NTP was at 100 $m\mu$ moles per 0.25 ml under standard conditions with 49 $m\mu$ moles DNA-P as primer. With four NTP 100 per cent incorporation was 1.39, 1.25 and 0.72 $m\mu$ moles with calf thymus (■—■), *T. pyriformis* (x—x), and *M. lysodeikticus* (□—□) DNA respectively. With ATP, GTP and CTP, 100 per cent equals 1.35 $m\mu$ moles with calf thymus (●—●), and 0.61 $m\mu$ moles with *M. lysodeikticus* DNA (o—o). With crab dAT (▲—▲), present at 0.025 O.D. units (260 $m\mu$), and synthetic dAT (Δ--Δ), present at 0.085 O.D. units (260 $m\mu$), 100 per cent incorporation with ATP and UTP were 1.21 and 0.99 $m\mu$ moles respectively.

calf thymus, *T. pyriformis* and *M. lysodeikticus* DNAs has its counterpart in the exchange reactions directed by the same DNAs. This difference is most striking at higher concentrations of actinomycin. With calf thymus DNA, a substantial residual exchange activity persists at actinomycin concentrations (50 μ g/ml and 100 μ g/ml) which totally suppress the same reactions with *M. lysodeikticus* DNA. This residual activity might be due to the presence of relatively long guanine-free sequences in calf thymus DNA which would not bind actinomycin and would thereby remain capable of priming the exchange reaction. In order to test this possibility, the four NTPs were separated by paper electrophoresis after an incubation in the presence of calf thymus DNA, actinomycin (50 μ g/ml) and PP³². All four NTPs were radioactive. Therefore, if guanine-free sequences are responsible for the exchange remaining at higher concentrations of actinomycin,

the priming of this residual exchange reaction violates the base-pairing principles which seem to govern the synthetic reaction. An alternative possibility is that some guanine-containing sequences may bind actinomycin very poorly or not at all. Such sequences may be present in higher frequency in calf thymus DNA compared with *M. lysodeikticus* DNA and prime the exchange reaction of all four NTPs even at very high concentrations of actinomycin.

All combinations of NTPs—singly, as well as all possible mixtures of equal concentrations of two, three and four NTPs—have been found to be inhibited by actinomycin in the exchange reaction. At 100 μg actinomycin/ml the exchange with each NTP singly was inhibited more than 90 per cent. However, the significance of the varying degrees of inhibition of exchange with the different individual nucleotides is difficult to assess since the rate of the reaction is markedly affected, for each nucleotide, by variations in its concentration and in the total NTP concentrations.

(g) Exchange reaction directed by dAT and dGC.—The data in Figure 7 show that the rates of exchange of individual NTPs with pyrophosphate need not reflect base composition of the priming DNA. As shown in Figure 6, the exchange reaction directed by dAT is totally resistant to

Figure 7. Effect of NTP concentration on NTP-PP³² exchange with either UTP or ATP alone, crab dAT (0.025 O.D. units 260 $\text{m}\mu/0.25$ ml) as primer. The exchange is expressed as $\text{m}\mu$ moles per ml reaction volume. Standard reaction conditions.

actinomycin, as is the synthetic reaction. The rate of the exchange reaction in the presence of both UTP and ATP when polynucleotide synthesis is proceeding, is substantially lower than with the equivalent concentrations of UTP alone, but these conditions permit an exchange of pyrophosphate with ATP which is much greater than that (Figure 7) with ATP alone. The effect of actinomycin on exchange reactions primed by dGC is shown in Figure 8. The exchange reaction with GTP alone is less sensitive to actinomycin than with CTP alone, resembling the corresponding homopolymer formation. Exchange reactions, with one exception, appear considerably more resistant to actinomycin than the homologous synthetic reactions. The single exception is the ex-

change with CTP alone primed by *M. lysodeikticus* DNA, whose actinomycin sensitivity closely parallels that of poly C formation primed by the same DNA.

DISCUSSION

Any hypothesis concerning the mode of action of actinomycin should be required to accommodate the following facts: (a) actinomycins which inhibit RNA-polymerase do so by binding to DNA; derivatives which are not bound fail to inhibit RNA polymerase and are biologically inactive; (b) DNAs (synthetic dAT) lacking guanine and cytosine do not bind biologically active actinomycins and prime RNA synthesis with complete immunity to actinomycin; (c) crab dAT resembles synthetic dAT but contains approximately 2.5 per cent GC; it binds actinomycin slightly,

Figure 8. Effect of actinomycin on NTP-PP³² exchange with either CTP or GTP, in the presence of either dGC or *M. lysodeikticus* DNA. The standard reaction conditions were modified by increasing the period of incubation to 30 minutes and by increasing the concentration of the individual NTP to 4 $\mu\text{moles/ml}$. The primer concentrations were 1.11 $\text{m}\mu\text{moles}$ dGC in 0.1 ml, and 49 $\text{m}\mu\text{moles}$ *M. lysodeikticus* DNA in 0.25 ml reaction volume. 100 per cent incorporation was 0.171 and 0.128 $\text{m}\mu\text{moles}$ with GTP and CTP respectively (dGC primer); and 0.175 and 0.429 $\text{m}\mu\text{moles}$ with GTP and CTP respectively (*M. lysodeikticus* DNA primer). *M. lysodeikticus* DNA priming of the exchange reaction is much less efficient and more actinomycin-sensitive than the dGC.

and its priming of RNA synthesis is inhibited by actinomycin; (d) apurinic DNA does not bind actinomycin, whereas apyrimidinic DNA does, though less efficiently than the corresponding native DNA; (e) dGC polymer binds actinomycin, and RNA synthesis primed by dGC is sensitive to ac-

tinomycin; (f) a portion of DNA-dependent poly A synthesis is resistant to actinomycin, as is oligothymidylate-directed poly A formation,⁹ whereas poly C and poly G formation is completely suppressed by actinomycin; (g) the ability of different naturally-occurring native DNA preparations to bind actinomycin reflects their guanine content.

Not all sequences containing guanine showed equal affinity in binding actinomycin or equal susceptibility to its inhibitory action on the priming of RNA synthesis. The binding effectiveness for actinomycin of a given DNA preparation could be directly correlated with its actinomycin susceptibility in directing the NTP-PP³² exchange. Since the exchange reaction can proceed with single NTP independently of concurrent polynucleotide synthesis it is likely that many short segments of DNA containing the requisite complementary bases can serve as primers of exchange. The relative actinomycin-resistance of the exchange reaction compared with that of the synthetic reaction is therefore not unexpected, since a scattering of bound actinomycin molecules which would prevent the formation of acid-insoluble polynucleotides could still leave significant areas of the DNA free to direct the pyrophosphate exchange.

An unexpected finding is the failure of dAT to prime an exchange reaction with ATP in the absence of UTP. This fact may have physiological significance. The sequence of nucleotides in dAT is known to be a perfectly alternating one of de-oxyadenylic and thymidylic acids.¹⁸ It is apparent that in this sequence pattern ATP cannot interact with the enzyme and primer to exchange its terminal pyrophosphate unit. However, such interaction is possible with either calf thymus or *M. lysodeikticus* DNA where the priming thymidylate residues presumably exist in all possible sequence patterns and the same interaction can take place with dAT provided UTP is present and polyribonucleotide chain growth is proceeding. It is possible that with dAT only UTP can serve to initiate polyribonucleotide chain growth. Thus the failure of ATP to exchange with PP³² in dAT-primed reactions may represent a model for visualizing DNA sequences which establish the starting point of RNA template synthesis under normal conditions. The ability of a given NTP to initiate polyribonucleotide chain growth may be determined not merely by the presence of its complementary base in the primer, but by the sequence of which such a base forms only one part.

The following conclusions can be drawn: (1) the presence of guanine in a polydeoxyribonucleotide is indispensable for actinomycin binding to DNA and for inhibition of RNA synthesis. The finding⁷ that adenine compounds may also cause spectral shifts in actinomycin solutions appears unrelated to the binding of actinomycin by DNA. (2) While guanine in a DNA preparation is required for actinomycin binding, optimal binding is favored by the native helical configuration. (3) Since dGC-directed reactions are more resistant to actinomycin than those directed by calf thymus or *M. lysodeikticus* DNA, it appears likely that optimal binding is influenced by the nucleotide sequences in the immediate environment of the guanine residues, perhaps in the contralateral strand, and that long sequences of deoxyguanylic acid do not present the most effective binding sites for actinomycin. This conclusion is also supported by the actinomycin-sensitivity of *M. lysodeikticus* DNA-directed poly C formation. (4) Since apurinic DNA does not bind actinomycin while dGC does, actinomycin binding to dGC probably involves only one strand—the polydeoxyguanylic strand. The independence of dGC-directed poly G and poly C formation suggests that RNA-polymerase replicates each strand of the primer independently. However, actinomycin can block both poly G and poly C formation directed by dGC. Therefore, actinomycin bound to only one strand of the primer can inhibit the synthesis of polyribonucleotides from both strands.

This may be due to the bulky peptide chains of actinomycin which may also account for the fact that this agent can suppress RNA synthesis much more effectively than proflavin.² (5) DNAs of high AT content prime the synthesis of poly A and poly U, but not of poly C, whereas M. lyso-deikticus DNA (74 per cent GC) and dGC can prime the formation of poly C but not poly A or poly U. Therefore, ribonucleotide homopolymer formation is likely to reflect complementary homopolymeric regions in the primer. The partial sensitivity to actinomycin of poly A and poly U synthesis suggests that these regions may vary in length and contain an occasional binding site of low affinity for actinomycin requiring a high concentration of the antibiotic for its saturation. Alternatively, the high concentrations of actinomycin required for inhibition of homopolymer synthesis might act by inducing profound configurational distortions in the primer.

Although some experiments supporting the above conclusions have been performed with concentrations of actinomycin higher than required to inhibit the formation of acid-insoluble poly-ribonucleotides, the effects of these concentrations retain the full specificity of actinomycin action. The relative actinomycin-resistance of the NTP-PP exchange is not unexpected, since concentrations of the antibiotic which could prevent the growth of polynucleotide chains to the size required for acid-insolubility would still leave many sequences free for priming the exchange reaction. Acid-soluble oligonucleotides may be formed under these conditions.

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COMPARATIVE UTILIZATION OF PSEUDOURIDINE TRIPHOSPHATE AND
URIDINE TRIPHOSPHATE BY RNA POLYMERASE*

By

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The relatively large amounts of pseudouridine in that fraction of the RNA concerned with the transfer of activated amino acids to ribosomes for peptide bond formation¹⁻⁴ suggests that this unusual base may play an essential role in this reaction, and that a specific mechanism exists for its incorporation into interribonucleotide linkage. To obtain information on the nature of this specificity, we have prepared Ψ UTP,^{†5} and studied its incorporation into RNA⁶ by a preparation of RNA polymerase obtained from nuclear extracts of HeLa cells.⁷ These experiments revealed that Ψ UTP can substitute with comparable efficiency for UTP in the synthesis of RNA from the four nucleoside triphosphates. When labeled in its innermost phosphate,** the P^{32} of Ψ UTP³² is carried into interribonucleotide linkage as the pseudouridyl nucleotide without prior conversion or transfer to a uridyl compound. The labeling pattern of the 2'(3') nucleoside monophosphates released upon alkaline hydrolysis of the synthesized radioactive RNA (expressed as the ratio of radioactivity associated with each particular 2'(3') nucleoside monophosphate compared with 2'(3') AMP taken as 1.00) is identical to that found with UTP³². The addition of Ψ UTP to the four required nucleoside triphosphates (including UTP) did not stimulate the rate of RNA synthesis. However, study of the labeling pattern produced in the presence of both Ψ UTP and UTP, one P^{32} -labeled and the other not, revealed pronounced changes in the ratios of alkali released P^{32} -2'(3') nucleoside monophosphates. These changes were dependent on the relative concentrations of the isomeric nucleoside triphosphates and were in opposite directions depending on which of the isomers was used as the radioactive compound. The data could be interpreted as indicating a preference on the part of the incorporating pseudouridylic acid to be linked through its 5'-phosphate group to the 3'-hydroxyl group of purine nucleosides, while uridylic acid was linked preferentially to pyrimidine nucleosides. The fact that no changes in the labeling pattern with CTP³² or ATP³² were found when both unlabeled isomers were present at different relative concentrations, is against the selection of a new DNA template upon addition of Ψ UTP and UTP.

This report describes experiments designed to determine the relationship of this phenomenon to the nature of the DNA which directs the synthesis of the RNA. The association of relatively large amounts of endogenous DNA with the HeLa cell RNA polymerase preparation makes

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[‡] The abbreviations used are: Ψ UTP, Ψ UDP, Ψ UMP, the 5'-triphosphate, -diphosphate and -monophosphate of pseudouridine (5-ribosyluracil); 5-FUTP, 5-fluorouridine triphosphate; DEAE-cellulose, diethylaminoethyl cellulose (standard); TCA, trichloroacetic acid; TEAC, triethylammoniumcarbonate.

** All P^{32} nucleotides used in this paper are labeled in the proximal 5'-phosphate only.

it unsuitable for these studies. They were therefore carried out using the partially purified RNA polymerase of microbial origin which is totally dependent on the addition of exogenous DNA for activity. The same labeling pattern phenomena have been observed despite the use of DNA's differing widely in base composition and secondary structure. Furthermore, 5-FUTP has been found to behave like UTP in this regard and not like Ψ UTP. Some of these results have been reported in preliminary form.⁸

EXPERIMENTAL PROCEDURE

Materials

Substrates— isolation of pseudouridine from urine. Initially Method I was used but more recently on improved, simplified procedure (Method II) based on prior desalting of the urine has been adopted.

Method I: 20 liters of urine from patients with leukemia, polycythemia or gout^{9,10} was adjusted to pH 10.8 with concentrated ammonium hydroxide, filtered, and passed through a 60 x 5 cm Dowex-1-acetate (x8, 100-200 mesh) column. Urine from healthy humans may also be used, although yields are lower. The column was washed with H₂O until the eluate was of neutral pH, and the pseudouridine then eluted with 0.05 M acetic acid. The elution was followed by observing for the characteristic alkaline bathochromic spectral shift.¹¹⁻¹³ Those fractions which showed A_{280}/A_{260} at pH 13.0 significantly above that at neutral pH, were combined. The product usually appeared in the 4th to 6th liter of eluate. The fractions were adjusted to pH 10.8 with ammonium hydroxide and again passed over a Dowex-1-acetate column (25 x 4 cm), washed with water, and eluted with 0.05 M acetic acid. The enriched eluates were evaporated to dryness in vacuo, dissolved in a minimum volume of water and subjected to preparatory chromatography on Whatman No. 3 MM paper using solvent system A. The product, identified by mobility of authentic marker pseudouridine and by its spectral properties, was eluted with water and subjected to rechromatography in the same system. Purification was completed by chromatography with solvent system B using Whatman No. 3 MM paper which had been washed extensively with acetic acid and ethanol. The pseudouridine band was cut off, eluted, adjusted to pH 10.8 with ammonium hydroxide, applied to a Dowex-1-acetate column (20 x 2 cm), and eluted with 0.05 M acetic acid. This procedure removes residual yellow impurities from the paper. The acetic acid was removed in vacuo and the product dissolved in water. After lyophilization, a fine white fluffy powder is obtained which is pure optically and 60-80 per cent pure pseudouridine by weight. Removal of any contaminating salts may then be accomplished by an additional paper chromatography in solvent system C. The final product is homogeneous in chromatographic systems A, B, C and D.

Method II: Desalting of the urine with Dowex 50 cation and Amberlite LA-2 (Rohm and Haas) liquid anion exchange resin was suggested by Dr. Waldo Cohn. Two to 3 liters of urine were passed through a Dowex 50, H⁺ form (8x, 100-200 mesh) column, 60 x 5 cm. The decolorized acid eluate was immediately mixed with Amberlite LA-2 liquid resin and the immiscible phases allowed to separate. The neutral opalescent lower aqueous layer was adjusted to pH 10.8 with concentrated ammonium hydroxide and applied to a Dowex-1-acetate (8x, 100-200 mesh) column, 35 x 5 cm. Three or four desalted aliquots of urine may be applied to one column, which is then washed with 2-3 liters of water, or until the optical density at 260 m μ falls below 0.05. Elution is then effected with 0.05 N acetic acid until the optical density at 260 m μ reaches 0.25. The eluate is evaporated to dryness in vacuo and the residue dissolved in a minimal volume of water.

The small amount of LA-2 resin carried along can now be removed by centrifugation. Paper chromatography in solvent system C is performed for 36 hours, approximately 400 optical density units at 260 m μ being applied to each sheet of washed paper. Excellent separation of pseudouridine is achieved with this single chromatographic separation. Paper impurities are removed by absorption on a small Dowex-1-acetate column and elution with 0.5 N acetic acid as described above. The acetic acid is removed by evaporation, and the pseudouridine is dissolved in water and then lyophilized. The white fluffy product is 80 per cent pure pseudouridine by weight assuming a_m to be 8.6×10^3 at 260 m μ , pH 7, and is homogeneous on paper chromatography in solvent systems A, B, C and D. A_{260}/A_{280} at pH 12 is 2.0. The yield of pseudouridine per 24-hour collection is about 50 mg.

Synthesis of Ψ UMP and Ψ UTP. A brief description of the methods used to prepare Ψ UMP and Ψ UTP, as well as proof of structure and spectral characteristics of these compounds, has already been reported.⁵ Elaboration on only certain additional points will be included here. Initial exploratory experiments on pseudouridine nucleotide synthesis were performed with a sample of pseudouridine isolated from urine by Dr. S. B. Weiss and purified by crystallization from aqueous ethanol by Dr. D. Rammler. Pseudouridine was converted to its 2'(3') 0-isopropylidene derivative by refluxing overnight in anhydrous acetone with zinc chloride as catalyst.¹⁴ Following complete removal of zinc ions with the ammonium form of IR 120 cation exchange resin, the product was isolated by chromatography on Whatman No. 3 MM paper in solvent system C. The isopropylidene derivative, separated from unreacted nucleoside and salt, was eluted with water and evaporated *in vacuo*, care being taken to avoid acidification. To insure complete removal of any contaminating strong base for the diester forming reaction, the isopropylidene nucleoside was passed over a small column of Dowex 50 (pyridinium) prior to use. Phosphorylation of the 5'-hydroxyl function was then accomplished with 2-cyanoethyl phosphate according to the method of Tener.¹⁵ In the preparation of unlabeled nucleoside monophosphate, two molar equivalents of phosphorylating agent were used to one of the protected nucleoside. This ratio was reversed in the synthesis of P^{32} -labeled nucleotide. After mild acid-catalysed removal of the isopropylidene protecting group (10 per cent acetic acid, 100° for 90 min), and mild alkaline elimination of the cyanoethyl group (9 M ammonium hydroxide, 60° for 90 min), the product was isolated by chromatography on DEAE-cellulose (carbonate). Elution was effected with TEAC, pH 7.5, using an exponential gradient from 0.02 M to 0.25 M.¹⁶ Pseudouridine 5'-phosphate appears at approximately 0.10 M TEAC. The yield of 5'-nucleotide was about 80 per cent with respect to the limiting reactant.

The mononucleotide was converted to the triphosphate using an enzyme derived from a yeast extract (see below). The Ψ UTP was isolated in 60 per cent yield by chromatography on DEAE-cellulose (carbonate), using a gradient of TEAC (pH 7.5) from 0.02 M to 0.4 M (Figure 1). Ψ UTP is eluted just before ATP in this system.

Synthesis of UTP^{32} and ATP^{32} . UTP^{32} and ATP^{32} were prepared in the manner described above for Ψ UTP³² using 2'(3') 0-isopropylidene uridine and 2'(3') 0-isopropylidene adenosine obtained from Aldrich Chemical Company, Inc., as starting materials.

Synthesis of 5-FUTP and 5-FUTP³². These compounds were prepared as described for the pseudouridyl nucleotides from 5-fluorouridine which was a gift of Dr. Scott of Hoffman-La Roche, Inc.

DNA preparations. Calf thymus DNA was obtained from Sigma Chemical Company; HeLa

cell DNA was prepared from cells in the logarithmic phase of growth in suspension culture by the method of Kay *et al.*;¹⁷ DNA from *Micrococcus lysodeikticus* was prepared by the method of Marmur;¹⁸ ϕ X-174 DNA was the gift of Dr. R. Sinsheimer.

Actinomycin C₁ (D) was obtained from Merck, Sharp and Dohme Research Laboratory Inc.;

Figure 1. DEAE-cellulose (carbonate) chromatography of Ψ UMP³² kinase reaction. The incubation mixture contained 5×10^{-2} M Tris, pH 7.5; 5×10^{-3} M MgCl₂; 3×10^{-2} M NaF; 10^{-2} M phosphoenolpyruvate; 4×10^{-4} M ATP; 7×10^{-4} M Ψ UMP³² (1.6×10^6 cpm per μ mole); and 0.1 mg pyruvate kinase and 8 mg of a dialyzed 50-75 per cent ammonium sulfate yeast fraction per ml of mixture. The final volume was 50 ml. Incubation was at 37° for 3 hours. Chromatography on DEAE-cellulose (carbonate) was performed using a column of 30 x 3 cm. A mixing chamber of 1000 ml containing 0.02 M TEAC, pH 7.5, and a reservoir containing 0.40 M of the same buffer. Each tube contained 7.0 ml of eluate.

phosphoenolpyruvate as the crystallized tricyclohexylammonium salt from California Corporation for Biochemical Research; and DEAE-cellulose from Brown and Company. GTP, ATP, CTP, and UTP were obtained as their sodium salts from Pabst Laboratories.

Chromatographic systems. Paper chromatography was carried out in the following solvent systems: A. isopropanol-ammonia-H₂O (7 : 2 : 1 volume for volume); B. isobutyric acid-0.5 M ammonium hydroxide (10 : 6 volume for volume); C. butanol-H₂O (86 : 14 volume for volume); D. isopropanolacetic acid-H₂O (7 : 2 : 1 volume for volume).

Enzymes—uridylic and pseudouridylic acid kinase. Dried brewer's yeast (Anheuser Busch,

Inc.) was autolyzed in 0.1 M sodium bicarbonate for 16 hours at 37°. The clear yellow supernatant fluid obtained after centrifugation at 18,000 x g for 20 minutes was fractionated with ammonium sulfate (Mann, enzyme grade). The 50-70 per cent ammonium sulfate fraction was collected by centrifugation at 18,000 x g for 15 minutes, and dialyzed against 0.05 M Tris, pH 7.5. This enzyme preparation contained both uridylic and pseudouridylic acid kinase activity. The enzyme preparation as described by Lieberman *et al.*,¹⁹ which contains kinase activity for both isomeric nucleoside monophosphates, and is less contaminated with nucleotide degrading enzymes, was also used. A typical incubation reaction mixture is detailed in the legend in Figure 1.

RNA polymerase. The DNA-directed RNA polymerase was isolated from *Escherichia coli* B as described by Chamberlin and Berg.²⁰ A preparation of RNA polymerase from *M. lysodeikticus* (500-fold purified) was a gift of Dr. S. B. Weiss. Pyruvate kinase from rabbit skeletal muscle was obtained from California Corporation for Biochemical Research.

Methods

Assays. The standard assay conditions used with the RNA polymerase from *E. coli* B were those described by Chamberlin and Berg.²⁰ The activity of the RNA polymerase was determined by measuring the incorporation of P³² into acid-insoluble material from one proximally labeled nucleoside triphosphate (specific activity of 2-50 x 10⁶ cpm per μ mole), in the presence of the other three unlabeled nucleoside triphosphates. The reaction was stopped with TCA added to 5 per cent final concentration, following the addition of a mixture of 2 mg of bovine serum albumin and 2 mg of yeast RNA. The acid-insoluble precipitate was washed three times with 5 per cent TCA, transferred to a planchet with 5 N ammonium hydroxide, dried, and counted in a thin window gas flow counter. Incorporation in the absence of added DNA was less than 2 per cent of the complete system. The assay with the RNA polymerase from *M. lysodeikticus* was as described by Weiss and Nakamoto.²¹ Incubations were carried out for 10 min at 37° unless otherwise noted. When unlabeled isomeric nucleoside triphosphate is added to an enzymatic reaction at twice the concentration of the P³²-labeled nucleoside triphosphate, radioactivity incorporated into RNA is decreased about 55 per cent while overall RNA synthesis remains unchanged. The specific activity ($m\mu$ moles of P³² incorporated per mg enzyme protein per hour) was 4000-5500 for the *E. coli* preparation and 2400 for the *M. lysodeikticus* preparation.

Alkaline hydrolysis of P³²-labeled RNA. This procedure is based on that of Weiss and Nakamoto.²¹ After three, 5 per cent TCA washes, the acid-insoluble radioactive material was washed once with ethanol-ether (3:1 volume for volume) and the residue was extracted twice for 30 min with 1.0 ml of 10 per cent sodium chloride pH 8.0, in a boiling water bath. 0.5 mg of carrier yeast RNA was added prior to each extraction. The nucleic acid of the combined extracts was precipitated in the cold with two volumes of ethanol. The precipitate was dissolved in 2 ml of 0.01 N sodium hydroxide; 0.2 ml of 20 per cent potassium acetate pH 5, was added and the nucleic acid reprecipitated with two volumes of ethanol. This procedure was repeated until a constant low number of radioactivity counts was present in the supernatant aqueous ethanol. The final precipitate was freed of ethanol *in vacuo* in a dessicator, dissolved in 1 ml of 0.3 N potassium hydroxide, and incubated at 37° for 18 hours. The solution was then cooled in ice and brought to pH 1 with 35 per cent perchloric acid. The acid-soluble fraction was brought to pH 4 with KOH. The solution was evaporated *in vacuo* in stages, the insoluble potassium perchlorate being removed by centrifugation. The volume of solution was reduced to about 0.05 ml and spotted on

Whatman No. 3 MM paper for electrophoresis in 0.025 M citrate, pH 3.5. The separated 2'(3') mononucleotides were identified with the aid of an ultraviolet lamp, eluted with water, plated, dried, and counted in a thin-windowed gas flow counter. The counts associated with each 2'(3') mononucleotide were compared with 2'(3') AMP taken as 1.00.

Protein was measured according to Lowry *et al.*²² Nucleic acid concentrations were based on the phosphorus content determined according to Fiske and SubbaRow.²³

RESULTS

RNA Synthesis with *M. lysodeikticus* RNA Polymerase

Labeling pattern with HeLa cell DNA. The results described in Table 1 using the RNA polymerase of *M. lysodeikticus* and DNA derived from HeLa cells, resemble those found with the enzyme system from HeLa cells.⁶ When either UTP³² or Ψ UTP³² is used for incorporation into

Table 1

RATIO OF RADIOACTIVITY IN ALKALINE HYDROLYSIS PRODUCTS OF P³²-RNA SYNTHESIZED BY RNA POLYMERASE (*M. lysodeikticus*) PRIMED BY HeLa CELL DNA

The standard reaction conditions for *M. lysodeikticus* RNA polymerase were used except that the incubation volume was 2.0 ml. Each tube contained 54 μ g of enzyme protein. The concentration of non-radioactive isomeric nucleoside triphosphate was twice that of the labeled compound. Each tube also contained ATP, CTP, and GTP

Alkali-released 2'(3') nucleoside monophosphate	Radioactive nucleoside triphosphate			
	UTP ³²		Ψ UTP ³²	
	Non-radioactive isomeric nucleoside triphosphate		Non-radioactive isomeric nucleoside triphosphate	
	no addition	Ψ UTP	no addition	UTP
Up	1.34	2.82	1.34	0.94
Gp	0.61	0.50	0.56	0.54
Ap	1.00	1.00	1.00	1.00
Cp	0.92	1.55	0.93	0.65

RNA in the absence of unlabeled isomer, the ratios of the radioactivity of the 2'(3') nucleotides derived from the synthesized RNA are remarkably similar. However, the addition of twice as much unlabeled isomeric nucleoside triphosphate leads to significant changes in these ratios. The changes are of rather large magnitude and are in opposite directions from the two isomers. Addition of cold Ψ UTP to the system incorporating UTP³² leads to a marked increase in the ratio of P³² appearing in the neighboring 2'(3') uridylic and cytidylic acids. Conversely, when unlabeled UTP is added to a system containing Ψ UTP³², radioactivity appearing in the 2'(3') pyrimidine nucleotides is decreased compared with that in the 2'(3') purine nucleotides.

Labeling patterns with *M. lysodeikticus* and calf thymus DNA's. *M. lysodeikticus* DNA has a relatively high dGC content (74 per cent), whereas calf thymus (and HeLa cell) DNA has a relatively low dGC content (44 per cent).²⁴ Accordingly, it was of interest to determine what effect,

if any, the base composition of the directing-DNA might have on the labeling phenomenon observed in the presence of the two isomeric nucleoside triphosphates. The results of such experiments are recorded in Tables 2 and 3. With both DNA's, the labeling pattern found when only one isomeric nucleoside triphosphate is present in the enzymatic reaction closely resembles that found when only the other isomer is used as the radioactive incorporating nucleotide. This

Table 2

RATIO OF RADIOACTIVITY IN ALKALINE HYDROLYSIS PRODUCTS OF
P³²-RNA SYNTHESIZED BY RNA POLYMERASE (M. lysodeikticus)
PRIMED BY M. lysodeikticus DNA

Reaction conditions are as described in Table 1.

Alkali-released 2'(3') nucleoside monophosphate	Radioactive nucleoside triphosphate			
	UTP ³²		ΨUTP ³²	
	Non-radioactive isomeric nucleoside triphosphate		Non-radioactive isomeric nucleoside triphosphate	
	no addition	ΨUTP	no addition	UTP
Up	0.72	1.55	0.71	0.53
Gp	2.00	1.89	2.26	1.86
Ap	1.00	1.00	1.00	1.00
Cp	2.24	3.95	2.24	1.60

Table 3

RATIO OF RADIOACTIVITY IN ALKALINE HYDROLYSIS PRODUCTS OF
P³²-RNA SYNTHESIZED BY RNA POLYMERASE (M. lysodeikticus)
PRIMED BY CALF THYMUS DNA

Reaction conditions are as described in Table 1.

Alkali-released 2'(3') nucleoside monophosphate	Radioactive nucleoside triphosphate			
	UTP ³²		ΨUTP ³²	
	Non-radioactive isomeric nucleoside triphosphate		Non-radioactive isomeric nucleoside triphosphate	
	no addition	ΨUTP	no addition	UTP
Up	1.30	2.84	1.34	0.93
Gp	0.86	0.55	0.65	0.77
Ap	1.00	1.00	1.00	1.00
Cp	1.23	1.76	1.10	0.73

pattern is characteristic for each particular DNA and in close agreement with what can be calculated from published data.^{21,25} Similarly, when cold isomeric nucleoside triphosphate was included in the reaction at twice the concentration of its labeled isomer, the shifts in labeling pat-

tern found with the HeLa enzyme system and with M. lysodeikticus RNA polymerase and HeLa cell DNA, were again observed with both M. lysodeikticus and calf thymus DNA's. A three-fold increase in concentration of the labeled nucleoside triphosphate in the absence of cold isomeric nucleoside triphosphate, did not influence the labeling pattern.

Relation of time course of RNA synthesis and labeling pattern shifts. An experiment was performed to determine whether the labeling pattern shifts were present to the same extent early in the course of RNA synthesis, or whether the observed changes developed during the synthesis. The synthetic reaction was incubated at 25° and the reaction stopped at 5 min and 20 min. As can be seen in Table 4, the shift in labeling pattern found upon addition of cold UTP to ΨUTP^{32} was as fully developed at 5 min, when about 30 per cent of RNA synthesis had been completed, as at 20 min, when synthesis had reached a plateau.

Table 4

EFFECT OF TIME ON THE RATIO OF RADIOACTIVITY IN ALKALINE HYDROLYSIS
PRODUCTS OF P^{32} -RNA SYNTHESIZED BY RNA POLYMERASE
(M. lysodeikticus) PRIMED BY CALF THYMUS DNA

Reaction conditions are as described in Table 1 except that incubation was carried out for the indicated times at 25°.

Radioactive nucleoside triphosphate	Alkali-released 2'(3') nucleoside monophosphate	Time (minutes)		
		5 min		20 min
		Non-radioactive isomeric nucleoside triphosphate		Non-radioactive isomeric nucleoside triphosphate
		no addition	UTP	UTP
ΨUTP^{32}	Up	1.20	0.88	0.89
	Gp	0.58	0.59	0.76
	Ap	1.00	1.00	1.00
	Cp	0.99	0.61	0.61

RNA Synthesis with E. coli RNA Polymerase and Calf Thymus DNA

Influence of ratio of $\Psi\text{UTP}/\text{UTP}^{32}$ on the shift in labeling pattern. The ratio of $\Psi\text{UTP}/\text{UTP}^{32}$ was varied to determine its effect on the shift in labeling pattern (Table 5). The maximal shift was expressed at a $\Psi\text{UTP}:\text{UTP}^{32}$ ratio of four; further increase in this ratio resulted in little change in labeling pattern.

Substitution of 5-FUTP for UTP or ΨUTP . 5-FUTP was about 25-30 per cent as effective as UTP or ΨUTP when used as the fourth nucleoside triphosphate for RNA synthesis (Table 6). As shown in Table 7, the labeling pattern obtained with 5-FUTP³² resembled that found with UTP³² or ΨUTP^{32} . Kahan has reported similar findings.²⁶ Fluorouridylate was shown to have been incorporated as such by alkaline hydrolysis of the synthesized P^{32} -RNA, separation of the 2'(3')-"uridylate" component by paper electrophoresis in 0.025 M citrate buffer of pH 3.5 and paper electrophoresis of the eluted "uridylate" in 0.1 M borate buffer of pH 9.15. In the latter

Table 5

EFFECT OF RELATIVE CONCENTRATIONS OF Ψ UTP AND
AND UTP³² ON THE LABELING PATTERN

Standard conditions were used with the *E. coli* RNA polymerase. The incubation volume was 0.25 ml containing 4.5 μ g of enzyme protein and 44 m μ moles of calf thymus DNA. UTP³² was maintained at a concentration of 200 m μ moles/ml while the concentration of Ψ UTP was varied. ATP, CTP and GTP were at 400 m μ moles/ml. Incubation was for 30 min at 37°.

Ψ UTP/UTP ³²	2'(3') nucleoside monophosphate			
	Up	Gp	Ap	Cp
0	1.30	0.68	1.00	1.14
0.5	1.95	0.81	1.00	1.62
1	2.22	0.69	1.00	1.74
2	2.60	0.64	1.00	1.87
4	2.98	0.71	1.00	1.96
8	3.08	0.65	1.00	1.99
16	3.21	0.62	1.00	1.92

Table 6

COMPARATIVE UTILIZATION OF UTP, Ψ UTP AND
5-FUTP IN RNA SYNTHESIS

Standard assay conditions were used with the *E. coli* RNA polymerase. The incubation volume was 0.25 ml containing 4.5 μ g of enzyme protein and 44 m μ moles of calf thymus DNA. The specific activity of ATP³² was 2.15×10^6 cpm per μ mole and for UTP³², Ψ UTP³² and 5-FUTP³² was 4.79×10^6 cpm per μ mole. Each nucleoside triphosphate was at a concentration of 400 m μ moles per ml.

Experiment	Radioactive nucleoside triphosphate	Non-radioactive nucleoside triphosphates	P ³² incorporation into RNA (m μ moles)
1	ATP ³²	UTP, GTP, CTP	3.95
		Ψ UTP, GTP, CTP	3.71
		5-FUTP, GTP, CTP	0.96
2	UTP ³²	ATP, GTP, CTP	2.68
	Ψ UTP ³²	ATP, GTP, CTP	2.40
	5-FUTP ³²	ATP, GTP, CTP	0.80

system the fluoronucleotide migrates as the fastest component.²⁷ Investigation of the labeling patterns after addition of cold 5-FUTP to a reaction mixture containing either UTP³² or Ψ UTP³², or after the addition of either cold UTP or Ψ UTP to a reaction mixture containing 5-FUTP³², revealed that 5-FUTP simulated UTP and not Ψ UTP in its behavior (Table 7). Whereas addition of cold 5-FUTP to UTP³² did not produce a labeling pattern shift, addition of cold 5-FUTP to

Table 7
COMPARATIVE EFFECT OF UTP, Ψ UTP AND 5-FUTP ON
THE SHIFTS IN LABELING PATTERNS

Standard reaction conditions were used with the *E. coli* RNA polymerase with 4.5 μ g of enzyme protein and 44 m μ moles of calf thymus DNA in 0.25 ml. The labeled nucleoside triphosphate, ATP, CTP and GTP were at a concentration of 400 m μ moles/ml. The unlabeled "extra" nucleoside triphosphate was at 800 m μ moles/ml.

Radioactive nucleoside triphosphate	Alkali-released 2'(3') nucleoside monophosphate	Extra non-radioactive nucleoside triphosphate			
		no addition	UTP	5-FUTP	Ψ UTP
UTP ³²	Up	1.30		1.23	2.55
	Gp	0.82		0.74	0.77
	Ap	1.00		1.00	1.00
	Cp	1.06		0.93	1.74
Ψ UTP ³²	Up	1.31	0.96	0.95	
	Gp	0.68	0.90	0.79	
	Ap	1.00	1.00	1.00	
	Cp	0.96	0.78	0.73	
5-FUTP ³²	Up	1.20	1.20		3.42
	Gp	0.85	0.84		0.88
	Ap	1.00	1.00		1.00
	Cp	1.20	1.19		2.70

Ψ UTP³² produced the same shift as UTP. Similarly, the addition of UTP to 5-FUTP³² led to no change in labeling pattern, while addition of Ψ UTP increased the radioactivity associated with the 2'(3') pyrimidine nucleotides.

E. coli RNA polymerase and ϕ X 174 DNA. Each strand of the DNA primer may be replicated by RNA-polymerase independently, with a mixture of the homopolymers of deoxyguanylate and deoxycytidylate as primer, polycytidylate or polyguanylate formation could be obtained when either CTP³² or GTP³² was the only substrate.²⁸ The shifts in labeling patterns in the presence of both isomeric nucleoside triphosphates might be due to the existence of sites with greater specificity for one of the isomeric nucleoside triphosphates in only one of the two strands of the primer DNA, rather than to the existence of sites of both types in each strand. This type of mechanism could lead to synthesis of a transfer RNA made from one DNA strand containing pseudouridylate, and having a base composition essentially complementary to the RNA made from the other DNA strand. The latter RNA would presumably serve as template for peptide bond formation. It was thought that the use of single-stranded DNA such as that from bacteriophage, ϕ X 174,²⁹ which is a primer for RNA polymerase²⁰ in experiments similar to those above might provide information on these points. It seemed possible that this DNA strand might code for a RNA lacking special sites for pseudouridine. As seen in Table 8, the same shifts in labeling patterns were observed with the single-stranded DNA. We can conclude that both kinds of sites appear to be present in the single-stranded DNA of ϕ X 174.

Table 8

RADIO OF RADIOACTIVITY IN ALKALINE HYDROLYSIS PRODUCTS
OF RNA POLYMERASE (*E. coli*) SYNTHESIZED
P³²-RNA PRIMED BY ϕ X 174 DNA

Reaction conditions are as described in Table 7 except that the reaction volume was 0.5 ml containing 54 m μ moles of ϕ X 174 DNA and incubation was for 90 min at 37°.

Alkali-released 2'(3') nucleoside monophosphate	Radioactive nucleoside triphosphate			
	UTP ³²		Ψ UTP ³²	
	Non-radioactive isomeric nucleoside triphosphate		Non-radioactive isomeric nucleoside triphosphate	
	no addition	Ψ UTP	no addition	UTP
Up	1.08	2.21	1.12	0.87
Gp	0.56	0.58	0.53	0.69
Ap	1.00	1.00	1.00	1.00
Cp	0.70	1.19	0.70	0.47

Homopolymer formation with *E. coli* RNA polymerase and calf thymus DNA. Chamberlin and Berg²⁰ have reported that RNA-polymerase is capable of forming homopolymeric ribopoly-nucleotides of adenylic and uridylic acids when calf thymus DNA (relatively high dAT content) is used to prime the reaction. This has been confirmed by several laboratories.^{28,30} In addition, we have reported the synthesis of polycytidylic acid by RNA polymerase using *M. lysodeikticus* DNA (relatively high dGC content) as primer.²⁸ As has been reported, polyuridylyate synthesis is not as efficient as that of polyadenylate. Polypseudouridylyate formation appears to be comparable with that of its isomeric compound (Table 9). However, synthesis of polypseudouridylyate with polynucleotide phosphorylase from Ψ UDP proceeds at a reduced rate compared with polyuridylyate formation.* Polyfluorouridylyate, which has been made with polynucleotide phosphorylase³¹ does not appear to be formed to any significant extent with the RNA polymerase system.

Comparison of actinomycin inhibition of RNA synthesis from Ψ UTP³² and UTP³². Actinomycin C₁, (D), a polypeptide-containing antibiotic, is a potent inhibitor of DNA-dependent RNA synthesis with RNA polymerase preparations of mammalian³² and microbial origin.^{28,30,33} This antibiotic acts by binding to deoxyguanylate residues in the DNA.^{28,33} Figure 2 shows that RNA synthesis using Ψ UTP³² or UTP³² is comparably inhibited by actinomycin. Similar results have been obtained using the isomeric nucleoside triphosphates with different actinomycins and derivatives which have varying activities as inhibitors of the RNA polymerase reaction.³³ Furthermore, homopolymer formation with either Ψ UTP or UTP by the RNA polymerase system was comparably resistant to the action of actinomycin.²⁸

DISCUSSION

The data suggest that at least two species of sites, differing quantitatively in their relative

* L. Sasse, M. Rabinowitz, and I. H. Goldberg, manuscript in preparation.

Table 9

HOMOPOLYMER SYNTHESIS WITH *E. coli* RNA
POLYMERASE AND CALF THYMUS DNA

Reaction conditions are as described in Table 6 except that only the indicated radioactive nucleoside triphosphate was substrate.

Radioactive nucleoside triphosphate	Incorporation into RNA* (m μ moles)
ATP ³²	1.34
UTP ³²	0.40
Ψ UTP ³²	0.50
5-FUTP ³²	0.05

* Alkaline hydrolysis of the P³²-RNA and paper electrophoresis and chromatography of the products revealed that 98 per cent of the radioactivity was associated with the 2'(3') mononucleotide corresponding to the radioactive nucleoside triphosphate used in the enzymatic reaction. Since assay for incorporation into RNA was based on acid insolubility (in the presence of carrier RNA and serum albumin) and polyuridylic acid is poorly precipitated by acid, the absolute incorporation for UTP, Ψ UTP and 5-FUTP may not be quantitative.

Figure 2. Inhibition of RNA synthesis from UTP³² and Ψ UTP³² by actinomycin. Standard assay conditions were used with the *E. coli* RNA polymerase. The incubation volume was 0.25 ml containing 4.0 μ g of enzyme protein and 44 m μ moles of calf thymus DNA. ●—● UTP³² used as the labeled nucleoside triphosphate, o---o Ψ UTP³² as the labeled nucleoside triphosphate. The concentration of actinomycin C₁ was varied as indicated.

selectivity for pseudouridylylate or uridylylate, may exist in the RNA made by the RNA polymerase system. Sites with greater affinity for pseudouridylylate or uridylylate become apparent only when adequate concentrations of isomeric nucleotide are available to saturate "uridylylate" sites of lower isomer specificity. Although this effect has been demonstrated experimentally by changing the relative concentrations of the isomeric nucleoside triphosphates, this should not be interpreted as indicating that composition of the product RNA is controlled solely by the relative concentrations of the reactants. There is no evidence against the base-pairing of both isomers with the deoxyadenylate of the priming DNA. It is possible, however, that the choice of the isomeric ribonucleotide may be determined by steric or other factors. Perhaps for steric reasons deoxynucleotides neighboring deoxyadenylate in the DNA determine which of the two isomers is placed complementary to deoxyadenylate. Such a mechanism might explain the increase in frequency with which one isomer is 2'(3')-linked to purine and the other to pyrimidines. This effect could be either a direct expression of the DNA primer, or an indirect one manifested through the neighboring ribonucleotides of the complementary RNA. Such a proposition would not conflict with the well established principle that selection of complementary ribonucleotides is dependent on the hydrogen binding characteristics of purines and pyrimidines, based on the Watson-Crick structure for DNA.^{20,21,34-37} Of pertinence, however, may be the findings of Trautner, Swartz and Kornberg³⁸ which suggest that the incorporation of deoxyguanylate by DNA polymerase primed by deoxyadenylate-deoxybromouridylylate copolymer may not be due to hydrogen-bonding of guanine to 5-bromouracil.

Structurally 5-FUTP is more closely related to UTP than to Ψ UTP. Accordingly, it is not unexpected that its behavior is like that of UTP in the labeling experiments. These results are consistent with those of Champe and Benzer³⁹ who concluded from genetic experiments that 5-fluorouracil acts mainly by incorporation into messenger-RNA in place of uracil, there acting partially like cytosine.

Based on the similar labeling patterns and equal incorporation rates when only UTP or Ψ UTP is present, it is suggestive that all "uridylylate" sites are available when only one of the isomers is present for RNA synthesis, but that only a few sites exhibit a greater affinity for one or the other isomer when both are available. It is possible, however, as suggested by the plateau in the labeling pattern found above a certain ratio of unlabeled nucleoside triphosphate to its labeled isomer, that instead there is a small number of sites with absolute specificity for one or the other isomeric nucleoside triphosphate. The similar labeling pattern found with each of the isomers in the absence of the other is against such a situation unless, because of the small number of sites with absolute specificity, the overall effect is quantitatively insignificant. Furthermore, such a mechanism would require that there be skip zones in the RNA copying process upon omission of the other isomeric nucleoside triphosphate. Such a mechanism has been suggested in explanation of RNA homopolymer formation by RNA polymerase with primer DNA containing all four bases.²⁰

Although the experimental approach used in this report cannot provide a complete explanation of the observed phenomena, it does suggest that, at least in special kinds of RNA synthesis, Ψ UTP is not simply an analogue of UTP, as might be inferred from experiments on the formation and utilization of glycosyl derivatives of pseudouridylylate.^{40,41} Polypseudouridylic acid prepared from Ψ UDP with polynucleotide phosphorylase can form a helix with itself and also with

polyadenylic acid.* It is conceivable that the results described in this paper might be related to the hydrogen-bonding properties of the pseudouridine.

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GLYCOGEN SYNTHESIS AND OTHER REACTIONS OF
PSEUDOURIDINE DIPHOSPHATE GLUCOSE*

By

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Uridine diphosphate glucose is the essential intermediate in hexose transformations and in uridyl and glycosyl transfer reactions.¹ As an outgrowth of experiments on enzymatic RNA synthesis in which pseudouridine triphosphate effectively substitutes for UTP,²⁻⁴ it was of interest to determine whether pseudouridine diphosphate glucose could be enzymatically synthesized and undergo the series of reactions characteristic of its uridine counterpart. A preliminary report describing such findings has been published.⁵ This paper will elaborate on these results and describe the formation of glycogen from Ψ UDP-glucose.[†] Evidence is presented favoring the concept that in these reactions Ψ UDP-glucose acts as an analogue of UDP-glucose.

EXPERIMENTAL PROCEDURE

Materials

Substrates. Ψ UTP was synthesized enzymatically from Ψ UMP which had been chemically prepared by phosphorylation of the 5'-hydroxyl function of pseudouridine with 2-cyanoethyl phosphate.^{4,6} Ψ UTP³² labeled in its innermost phosphate was prepared in the same manner using p³²-labeled cyanoethyl phosphate.

Glucose-1-P and galactose-1-P were obtained from the Schwarz Biochemical Company; NAD, NADP, UTP, UDP-glucose and ATP from Pabst Laboratories; phosphoenolpyruvate, cyclohexylamine salt, from California Corporation for Biochemical Research; and glucose-1-C¹⁴-6-P, specific activity 1 μ c per μ mole, from the New England Nuclear Company. UDP-glucose-1-C¹⁴ was prepared from glucose-1-C¹⁴-6-P and UTP using reaction conditions identical to those described below for Ψ UDP-glucose-1-C¹⁴ synthesis. Ammonium sulfate was Mann "enzyme grade."

Enzymes. Dried brewers yeast (Anheuser Busch, Inc.) was autolyzed in 0.1 M NaHCO₃ for 16 hours at 37°. The clear yellow supernatant fluid obtained after centrifugation for 20 minutes at 18,000 g was fractionated with ammonium sulfate. The 0 to 50 per cent saturated fraction was dialyzed overnight against 20 volumes of 0.05 M Tris pH 8.1. This preparation was used for the enzymatic synthesis of Ψ UDP-glucose.

Rat liver UDP-glucose glycogen synthetase was prepared from 3-day starved animals according to the method of Steiner.^{7,8} Both particulate and soluble** enzyme preparations were

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[‡] Abbreviations: Ψ UMP, Ψ UDP and Ψ UTP are the mono-, di- and tri-phosphates of pseudouridine (5-riboseyluracil).

** Unpublished data of Dr. D. F. Steiner.

used. Crude galactose-1-P uridyl transferase was prepared by the method of Maxwell.⁹ UDP-glucose dehydrogenase (type 3), crystalline muscle phosphorylase and crystalline phosphoglucomutase was purchased from Sigma Chemical Company. Penicillium notatum glucose oxidase was obtained from Worthington Biochemical Corporation and pyruvate kinase from California Corporation for Biochemical Research.

The following paper chromatographic solvent systems were used: solvent A—ethanol, 0.5 M ammonium acetate, pH 7.5 (5:2 volume for volume); solvent B—ethanol, 0.5 M ammonium acetate, pH 3.8 (5:2 volume for volume); solvent C—ethyl acetate, H₂O-pyridine (2:2:1 volume for volume).

Phosphate was determined by the method of Fiske and SubbaRow,¹⁰ and glucose by oxidation with Penicillium notatum glucose oxidase. Paper electrophoreses were performed with the Durum-type apparatus in 0.02 M citrate buffer, pH 3.8 and 5.4. Spectra were recorded on a Cary 14 recording spectrophotometer.

RESULTS

Pseudouridine-containing nucleotides participate in the following reactions, reaction rates being 10-25 per cent of those obtained when the uridine counterparts were used.

- 1) $\Psi\text{UTP} + \text{glucose-1-P} \rightleftharpoons \Psi\text{UDP-glucose} + \text{PP}_i$
- 2) $\Psi\text{UDP-glucose} + \text{galactose-1-P} \rightleftharpoons \Psi\text{UDP-galactose} + \text{glucose-1-P}$
- 3) $\Psi\text{UDP-glucose} + 2 \text{ NAD} \rightleftharpoons \Psi\text{UDP-glucuronic acid} + 2 \text{ NADH}$
- 4) $\Psi\text{UTP} + \text{galactose-1-P} \rightleftharpoons \Psi\text{UDP-galactose} + \text{PP}_i$
- 5) $\Psi\text{UDP-glucose} + \text{glycogen} \rightleftharpoons \text{glycogen 1, 4 glucose} + \Psi\text{UDP}$

1) $\Psi\text{UDP-glucose pyrophosphorylase activity}$. $\Psi\text{UDP-glucose}$ was synthesized from ΨUTP and glucose-1-P using the enzyme preparation obtained from dried brewers yeast autolysate. Since this relatively crude enzyme preparation contained substantial nucleotide triphosphatase activity, the addition of the ATP generating system, phosphoenolpyruvate and phosphoenolpyruvate kinase, increased the yield of $\Psi\text{UDP-glucose}$ from 60 per cent to 90-95 per cent. The optimal reaction mixtures contained 1×10^{-3} M ΨUTP , 1×10^{-2} M glucose-1-P, 3×10^{-2} M Tris, pH 8.1, 5×10^{-3} M MgCl_2 , 2.5×10^{-3} M cysteine, 5×10^{-4} M ATP, and 5×10^{-3} M phosphoenolpyruvate. The 1.0 ml reaction mixtures containing 25 μg crystalline phosphoenolpyruvate kinase and 0.5-1.0 mg brewers yeast enzyme were incubated for 15 min at 37°. After boiling for 2 min, and centrifugation, the product was isolated from the supernatant fluid by initial chromatography in solvent A on Whatman No. 3 MM paper (Figure 1, cf. ¹), elution with water, and rechromatography in solvent B. By this means $\Psi\text{UDP-glucose}$, free of contaminating nucleotides or other reactants was obtained. In both systems A and B, the mobility of $\Psi\text{UDP-glucose}$ was 0.73 in reference to that of UDP-glucose. Following elution from the paper, residual ammonium acetate could be removed by repeated evaporations to dryness in vacuo.

P^{32} -labeled $\Psi\text{UDP-glucose}$ was made in the same manner using ΨUTP^{32} labeled in its innermost phosphate. $\Psi\text{UDP-glucose-C}^{14}$ was also prepared using identical reaction conditions except that glucose-C¹⁴-6-PO₄, specific activity 1 μc per μmole , replaced glucose-1-P and 1 μg of crystalline phosphoglucomutase was added. $\Psi\text{UDP-glucose}$ was also prepared with both the α phosphates labeled with P^{32} and glucose labeled with C^{14} .

The isolated product possessed the chemical composition and reactivity expected for $\Psi\text{UDP-glucose}$. Table 1 shows the analytic data based on a molar absorptivity index of 8.6×10^3 at 260

Figure 1. Autoradiogram of paper chromatograms developed in solvent system A of the products of acid and alkaline hydrolysis of P^{32} -labeled Ψ UDP-glucose.

A. hydrolysis for 15 min. in 1 N HCl at 100°.

B. hydrolysis for 15 min in 0.01 N HCl at 100°; hydrolysis for 15 min at 1 N NaOH at 100°.

m μ at pH 7.0.¹¹ Phosphate analysis after ashing with sulfuric acid¹⁰ yielded approximately 2 moles of phosphate per mole of pseudouridine. The glucose residue, released by hydrolysis for 15 min at 100° in 1 N HCl, was identified by paper chromatography in solvent C. The identity of the sugar was further confirmed by oxidation with the specific *Penicillium notatum* glucose oxidase. Using this method the molar ratio of glucose to pseudouridine was approximately 1:1.

Table 1
CHEMICAL ANALYSIS OF PSEUDOURIDINE NUCLEOTIDES

Nucleotide	Pseudouridine* μ moles	Phosphate μ moles	Glucose μ moles
UMP	1.0	1.06	-
UTP	1.0	3.05 (1.93) [†]	-
UDP-glucose	1.0	2.06	0.91 [†]

All values are averages of 3-6 analyses.

*Based on a_m at 260 m μ , pH 7.0 of 8.6×10^3 .¹¹

[†]Released after boiling in 1 N HCl for 7 min.

Ψ UDP-glucose behaved identically to UDP-glucose with reference to acid and alkaline hydrolysis. Figures 1A and 1B show autoradiograms of reaction products of acid and alkaline hydrolysis of P³²-labeled Ψ UDP-glucose. Heating at 100° for 15 min in 1 N HCl yields Ψ UMP³², milder hydrolysis in 0.01 N HCl at 100° gives Ψ UDP³², whereas alkaline hydrolysis in 1 M NaOH at 100° gives the reaction product Ψ UMP³².

The ultraviolet absorption spectrum of Ψ UDP-glucose has previously been published.⁵ It shows the alkaline bathochromic shift characteristic of pseudouridine compounds. The spectrum differs from that of the nucleoside in that the A_{280}/A_{260} m μ at pH 13 is 1.6 rather than 2.0 and the absorbance shows an increase at pH 14 rather than a decrease. The spectral characteristics are identical to those obtained with Ψ UMP, Ψ UDP, Ψ UTP.⁶

2) Galactose-1-P pseudouridyl transferase activity. Galactose-1-P pseudouridyl transferase activity was demonstrated both in rat liver and in human red cell hemolysates. A partially purified rat liver preparation of galactose-1-P uridyl transferase⁷ catalyzed a galactose-1-P dependent release of glucose-1-P. The glucose-1-P was converted to glucose-6-P by phosphoglucomutase and the reaction followed by observing NADP reduction with glucose-6-P dehydrogenase. Approximately 1 mole of NADP was reduced per mole of Ψ UDP-glucose added; omission of Ψ UDP-glucose or of galactose-1-P resulted in the formation of no NADPH. The relative rates of reaction with Ψ UDP-glucose and UDP-glucose are shown in Figure 2. The initial zero order rate with Ψ UDP-glucose was approximately 16 per cent of that with UDP-glucose at the same concentration.

Pseudouridyl transferase activity was also demonstrable in human red blood cell hemolysates. Plasma was removed from freshly-drawn heparinized blood and the cells washed in 0.9 per cent sodium chloride. The washed cells were hemolyzed in one volume of distilled water, and assayed for galactose-1-P pseudouridyl transferase activity, in a manner analogous to a method of Anderson *et al.*¹² After incubation of the hemolysate with Ψ UDP-glucose and

Figure 2. Relative rates of oxidation of Ψ UDP-glucose and UDP-glucose to the glucuronide derivative. The 1.0 ml reaction mixture contained 0.1 M glycine pH 8.75, 1×10^{-3} M NAD, 1.4×10^{-4} M Ψ UDP-glucose or UDP-glucose and 0.49 mg UDP-glucose dehydrogenase. Reduction of NAD was followed continuously at 340 m μ using a Cary Model 14 Recording Spectrophotometer.

galactose-1-P, the residual Ψ UDP-glucose was determined by oxidation of NAD with UDP-glucose dehydrogenase. Normal red cell hemolysates contain galactose-1-P pseudouridyl transferase activity equal to about 10 per cent of that of uridyl transferase. Hemolysates of galactosemic patients, in whom galactose-1-P uridyl transferase is specifically absent, similarly revealed no galactose-1-P pseudouridyl transferase activity (Table 2). This implies that both reactions are catalyzed by the same enzyme.

3) Ψ UDP-glucose dehydrogenase activity. The oxidation of Ψ UDP-glucose to Ψ UDP-glucuronic acid by NAD was obtained using a partially purified preparation of UDP-glucose dehydrogenase. Two moles of NAD were reduced per mole of glucose oxidized. A comparison of the reaction rates with Ψ UDP-glucose and UDP-glucose appears in Figure 3. The first order rate constants determined from the initial part of the NAD-dependent oxidation of Ψ UDP-glucose was approximately 22 per cent of that for UDP-glucose at identical substrate concentrations.

Chromatography of the reaction products revealed complete conversion of Ψ UDP-glucose- C^{14} to a compound which in solvents A and B moved below marker UDP-glucuronic acid, with relative R_f values in both systems of 0.74. Acid hydrolysis of the eluted C^{14} -labeled nucleotide yields glucuronolactone- C^{14} and glucuronic acid- C^{14} identified by paper chromatography in butanol-acetic acid H_2O (3:1:1, volume for volume).

4) Ψ UDP-galactose pyrophosphorylase activity. Ψ UDP-galactose pyrophosphorylase activity was shown to be present in the crude yeast autolysates. The formation of Ψ UDP 32 -galactose was demonstrated by chromatography in solvents A and B. Reaction conditions were identical to that used for formation of Ψ UDP-glucose, except that 1×10^{-2} M galactose-1-P replaced glucose-1-P.

Table 2
GALACTOSE-1-PO₄ URIDYL AND PSEUDOURIDYL TRANSFERASE
ACTIVITIES IN RED CELL HEMOLYSATES OF NORMAL
AND GALACTOSEMIC PATIENTS

Patient	Age-Sex	UDP-glucose (μ moles converted in 90 min)	UDP-glucose (μ moles converted in 30 min)
Normal	4 yr.♂	0.030	0.102
Normal	3 yr.♂	0.029	0.138
Galactosemic	4 yr.♂	0.000	0.001
Galactosemic	6 yr.♀	0.000	0.002

Duplicate 1.0 ml reaction mixtures contained 90 μ moles Tris buffer, pH 8.1, 0.23 μ moles UDP-glucose or Ψ UDP-glucose and 0.3 ml of red blood cell hemolysate. 0.4 μ moles galactose-1-P were added to one duplicate tube. Reactions with UDP-glucose were incubated for 30 min and at 37° and those with Ψ UDP-glucose for 90 min. The reaction was stopped by boiling for 2 min after the addition of 1.5 ml H₂O.

The amount of Ψ UDP-glucose or UDP-glucose remaining after incubation was determined by NAD reduction with UDP-glucose dehydrogenase.¹² The decrease in the amount of Ψ UDP-glucose or UDP-glucose in the presence of galactose-1-P represents the conversion to the galactose-containing nucleoside diphosphate compound.

Figure 3. Relative rates of galactose-1-P pseudo-uridyl and galactose-1-P uridyl transferase activities using rat liver enzyme. The 1.0 reaction mixture contained 0.1 M Tris, pH 8.7, 1×10^{-2} M MgCl₂, 1×10^{-3} M cysteine, 1×10^{-3} M NADP, 5×10^{-4} M galactose-1-P, 2×10^{-4} M Ψ UDP-glucose or UDP-glucose, 0.5 mg galactose-1-P uridyl transferase and 0.1 mg glucose-6-P dehydrogenase. The reaction was followed continuously at 350 m μ using a Cary Recording Spectrophotometer Model 14. The galactose-1-P pseudouridyl transferase reaction was rate limiting as shown by a very rapid reduction of NADP upon the addition of glucose-1-P.

5) Glycogen synthesis from Ψ UDP-glucose. Glucose- C^{14} incorporation into glycogen from Ψ UDP 32 -glucose- C^{14} was shown using soluble and particulate preparations of glycogen synthetase purified approximately 200-fold from rat liver.^{7,8} Paper chromatography with solvent A showed that glycogen- C^{14} and Ψ UDP 32 were the only products of this reaction (Figure 4). The labeled glycogen was isolated by repeated alcohol precipitation following heating for 10 min at

Figure 4. Autoradiogram of a paper chromatogram developed in solvent system A of products of the reaction synthesizing glycogen from Ψ UDP-glucose. The 50 μ l reaction mixture contained 1×10^{-3} M Ψ UDP-glucose labeled with P^{32} in its innermost phosphate (specific activity of 7.5×10^5 cpm per μ mole) and with glucose- C^{14} (specific activity of 4×10^6 cpm per μ mole), 0.1 M Tris, pH 7.5, 0.5 mg glycogen, 1×10^{-2} M glucose-6-P, and 10 μ g particulate rat liver glycogen synthetase. The mixture was incubated at 27° for 15 min, heated to 100° for 2 min, and the supernatant fluid applied to Whatman No. 3 MM paper for chromatography.

100° in 30 per cent KOH. It was shown to consist of 1, 4-linked glucose residues by reaction with β amylase to form C^{14} -labeled maltose.¹³ Phosphorolysis of the radioactive glycogen with crystalline muscle phosphorylase yielded C^{14} -labeled glucose-1-P.

A comparison of the rates of incorporation into glycogen of glucose- C^{14} from Ψ UDP-glucose- C^{14} and UDP-glucose- C^{14} , is seen in Figure 5. Glycogen synthesis was 10 to 15 times slower

with Ψ UDP-glucose- C^{14} than with UDP-glucose- C^{14} . Contamination of the enzyme with amylase is probably responsible for the incomplete transfer of glucose- C^{14} to glycogen, and for the decreased incorporation from UDP-glucose- C^{14} at longer incubation periods.

Figure 5. Time course of per cent incorporation of glucose- C^{14} into glycogen from Ψ UDP-glucose- C^{14} and UDP-glucose- C^{14} . The 30 μ l reaction mixture contained 0.1 Tris sulfate, pH 7.4, 1.3×10^{-2} M glucose-6-phosphate, 8×10^{-3} M EDTA, 0.85×10^{-3} M Ψ UDP-glucose- C^{14} or 0.77×10^{-3} M UDP-glucose- C^{14} , each of specific activity 2.9×10^5 cpm per μ mole, 0.3 mg glycogen, and particulate rat liver glycogen synthetase (15 μ g of protein). After incubation at 30° the reaction was stopped by the addition of 0.5 ml of 30 per cent KOH. Glycogen was isolated as described in Methods and counted in a windowless Geiger-Müller gas flow counter, corrections for self-absorption being made.

The slower rate of glycogen synthesis with Ψ UDP-glucose was not secondary to large differences in K_M for the two substrates. The K_M for Ψ UDP-glucose averaged 0.76×10^{-3} M (Figure 6) and for the UDP-glucose 0.37×10^{-3} M (Figure 7). Both reactions were inhibited competitively by the nucleoside diphosphate products of the reactions (Figures 6 and 7). UDP inhibited about 3 to 4 times more effectively than did Ψ UDP whether Ψ UDP-glucose or UDP-glucose was used as substrate (Table 2). Inhibition by ADP was of intermediate effectiveness. The similar patterns of inhibition by UDP and Ψ UDP with either substrate are compatible with the presence of only one glycogen synthetase enzyme in this partially purified preparation. Ψ UDP-glucose did not detectably inhibit glycogen synthesis from C^{14} -labeled UDP-glucose, in fact, a suggestion of stimulatory effect was seen in several experiments. In the converse reaction in which Ψ UDP-glucose- C^{14} was used as substrate, the inhibitory activity of UDP-glucose was found to be slightly less than that of Ψ UDP. A UTP-generating system was used here to remove the rapidly accumulating UDP.

Figure 6. Lineweaver-Burke plot of reciprocals of velocity and substrate concentration for glycosyl transfer to glycogen from Ψ UDP-glucose- C^{14} in the absence and presence of inhibitors. Assays were performed using standard conditions; the reactions were allowed to progress to no greater than 20 per cent of completion. 5×10^{-3} M phosphoenolpyruvate and $10 \mu\text{g}$ pyruvate kinase were added to those reactions in which UDP-glucose was used as inhibitor. Particulate rat liver enzyme was used.^{7,8}

▲—▲, no inhibitor added; ▽—▽, 0.95×10^{-3} M Ψ UDP; x—x, 0.91×10^{-3} M UDP-glucose; o—o, 0.95×10^{-3} M UDP.

Note the decreased velocity at high substrate concentration in each curve, probably indicative of substrate inhibition.

DISCUSSION

Although a specific mechanism may exist for the incorporation of pseudouridylic acid into transfer RNA, no evidence for the natural occurrence or the specific participation of Ψ UDP-glycosyl compounds in enzymatic reactions has been presented. Ψ UDP-glucose dehydrogenase, Ψ UDP-galactose pyrophosphorylase, and galactose-1- PO_4 uridyl transferase activities were all present in brewers yeast extracts; however, the rates of reaction were uniformly 10-25 per cent of those obtained with the respective uridyl compounds. Of special significance was the fact that in galactosemic patients, the pseudouridyl galactose-1-P transferase reaction was absent. A single genetically directed enzyme defect, the absence of galactose-1-P uridyl transferase, is believed to be present in galactosemia. The parallel disappearance of pseudouridyl and uridyl galactose-1-P transferase activities, therefore, provides strong evidence that the catalysis of both reactions is by a single enzyme.

Glycogen synthesis has been demonstrated by Goldemberg¹⁴ from ADP-glucose as well as from UDP-glucose. ADP-glucose was 50 per cent as active as UDP-glucose, but CDP-glucose, IDP-glucose and ADP-maltose did not serve as substrates for the enzyme. ADP-glucose, however, appears to be the most effective substrate in starch synthesis.¹⁵ The results described in

Figure 7. Lineweaver-Burke plot for glycosyl transfer from UDP-glucose-C¹⁴ to glycogen in the absence and presence of inhibitors. Assay was done as in Figure 6.

▲—▲, no inhibitors; x—x, 2×10^{-3} M UDP-glucose; ▽—▽, 0.36×10^{-3} M ADP; ■—■, 1.3×10^{-3} M Ψ UDP; ●—●, 0.5×10^{-3} M UDP; ○—○, 2.1×10^{-3} M UDP.

this paper suggest that one and the same enzyme is responsible for glycogen synthesis from UDP-glucose and Ψ UDP-glucose and that the pseudouridyl derivatives act as analogues of their uridine counterparts. It is still possible, although perhaps unlikely, that as in the case of ADP-glucose, in systems other than those studied here, the pseudouridine compounds may be the preferred substrates.

Table 3
INHIBITOR CONSTANTS OF GLYCOSYL
TRANSFER TO GLYCOGEN

Substrate	Inhibitor	K _i
UDP-glucose	UDP	0.25×10^{-3} M
	Ψ UDP	0.78×10^{-3} M
	ADP	0.44×10^{-3} M
	Ψ UDP-glucose	No inhibition
Ψ UDP-glucose	UDP	0.70×10^{-3} M
	Ψ UDP	3.5×10^{-3} M
	UDP-glucose	* 0.91×10^{-3} M

Assays were performed as indicated in Figures 6 and 7.

* Approximation, since most of the UDP-glucose is converted to UTP at the end of reaction.

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