worthy homology indicated by these results is that the *hi-4* region of yeast appears to have the same functional complexity as the *hist-3* region of *Neurospora* (*5, 10*).

G. R. FINK

Josiah Willard Gibbs Laboratories, Yale University, New Haven, Connecticut

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Replication of the RNA of Bacteriophage R17

Abstract. Escherichia coli *cells were irradiated with ultraviolet light to stop ribosomal RNA synthesis. After infection of such cells with the single-stranded RNA phage R17, the RNA most rapidly labeled by a pulse of tritiated uridine sedimented in a broad band in the 16S region of sucrose gradients. The effect of ribonuclease on this material and its behavior during a "chase" period in nonradioactive medium suggest that it consists of a core of double-stranded RNA, one strand of which—the viral strand—is continually displaced by a growing strand forming single-stranded tails and ultimately free 27S viral RNA.*

Molecules with some of the properties of double-stranded RNA (*1*) have been detected in both mammalian (*2, 3*) and bacterial (*4*) cells infected with RNA viruses, as well as in the product in vitro of virus-induced RNA polymerases from both bacterial and mammalian cells (*5*). That this double-stranded ribonuclease-resistant RNA is probably relevant to virus growth is implied by the observations that it is infective (*2*) and that much of the RNA of the the originally infecting virus is transformed to such a form (*4*). Our study concerns the formation of double-stranded RNA in *Escherichia coli* cells infected with the RNA phage R17, and the part played by it in the replication of the phage RNA.

Bacteriophage R17 and its host, *E. coli* K12 strain Hfr$_1$ (*6*), were grown in TPG minimal medium (*7*) supplemented with 0.5 percent glucose and 0.1 percent Bacto-tryptone (*8*).

Because these studies involve extraction of RNA from bacterial cells, it was first necessary to show that newly formed viral RNA is not degraded during lysis of the cells and extraction of the RNA. A sample of labeled R17 RNA was added to a suspension of *E. coli* cells, and RNA was extracted from the mixture and centrifuged through a sucrose gradient (Fig. 1). The viral RNA formed a peak of radioactivity just ahead of the 23*S* ribosomal RNA, at about 27*S*, in agreement with Ellis and Paranchych (*9*). There was very little radioactivity higher up the tube, an indication that the viral RNA was not appreciably degraded.

In cells infected with R17 a high rate of normal cellular RNA synthesis persisted. When tritiated uridine was added in 1- or 5-minute "pulses" 30 minutes after infection, there were labeled bands in the sucrose gradients at 4*S*, 16*S*, and 23*S* and a small additional peak or shoulder at 27*S*, indicating synthesis of viral RNA. However, when RNA from cells labeled between 30 and 35 minutes after infection was treated with pancreatic ribonuclease (1 μg/ml for 10 minutes at 37°C in 0.1*M* NaCl) and then analyzed in a sucrose gradient, a labeled peak remained which centered at fractions 17 to 18 (about 10*S*). There was no such material in uninfected cells.

Since we were interested in virus-specific labeled materials which might have been obscured in the gradients by the labeling of ribosomal RNA, we attempted to reduce the background rate of cellular RNA synthesis by irradiating the cells with ultraviolet light before infection, a technique which had been used previously to study RNA synthesis in animal cells infected with poliovirus (*10*). With increasing doses of ultraviolet light, the decrease in yield of infectious virus was about equal to the decrease in rate of synthesis of RNA (measured by the radioactivity in the fraction precipitable by trichloroacetic acid after a 5-minute pulse of tritiated uridine). Nevertheless, cells whose rate of total RNA synthesis had been reduced to about 5 percent of normal by ultraviolet irradiation

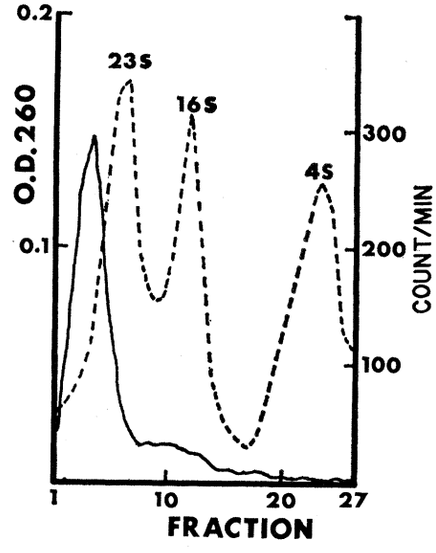

Fig. 1. Sedimentation pattern of a mixture of R17 and *E. coli* RNA. P^{32}-labeled R17 phage was shaken with phenol at 20°C and the phenol was removed by two extractions with ether. A sample of this labeled phage RNA was added to a suspension of *E. coli* (about 10^{10} cells) in 1 ml of solution (0.1*M* NaCl, 0.001*M* magnesium acetate, 0.05*M* tris buffer, *p*H 7.2). The cells were lysed by adding 1 percent sodium dodecyl sulfate, and then alternately frozen and thawed four times. The lysate was shaken vigorously with phenol for 1 minute at 20°C and centrifuged; RNA and DNA in the aqueous phase were precipitated by adding two volumes of ethanol at 0°C. The nucleic acids were sedimented (15 minutes at 5000 rev/min), redissolved, precipitated again with ethanol, and finally dissolved in 0.2 ml of a solution of 0.1*M* NaCl and 0.001*M* magnesium acetate. The nucleic acid solution was layered on a 4-ml sucrose gradient (7 to 20 percent) containing 0.1*M* NaCl and 0.001*M* magnesium acetate, and centrifuged in a Spinco SW 39 rotor for 4 hours at 37,500 rev/min at the 30°F temperature setting. Equal fractions of about 0.15 ml were then collected from the bottom of the tube, and 0.1 ml of each was transferred to 12 ml of scintillation fluid containing methanol and toluene at 3:10 and counted in a Packard Tricarb scintillation counter. The remainder of each fraction was diluted with 0.5 ml of water, and the optical density was measured at 260 mμ. Solid line: radioactivity (R17 RNA). Broken line: optical density (*E. coli* RNA).

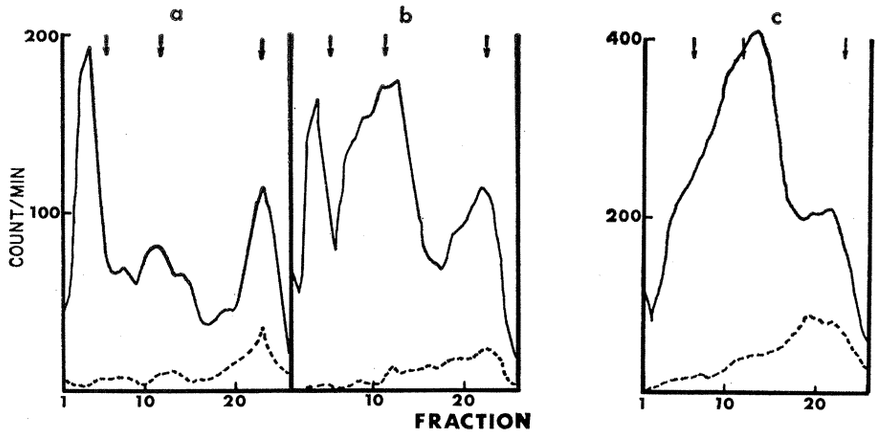

Fig. 2. RNA from ultraviolet-irradiated cells, with and without infection. Growing cultures at 2 to 5 × 10^8 cells per milliliter were centrifuged, and the cells were resuspended in 10 percent of the original volume of phosphate-buffered saline containing 0.001*M* $CaCl_2$. They were then irradiated in 2-ml quantities in a petri dish (9 cm in diameter) for 15 seconds at 20°C, at a distance of 15 cm from a GEC G8T5 8-watt lamp. After this treatment, approximately 10^{-5} of the original cells were able to form colonies on nutrient agar. To infect the cells, a small volume of R17 was added to give about 20 plaque forming units per cell, 5 minutes at 20°C being allowed for absorption. At zero time the infected cells were transferred to four volumes of growth medium at 37°C. Tritiated uridine (11.6 mc/mg, New England Nuclear Corp.) was added as follows: (i) 0.02 μc/ml for a 5-minute pulse, 25 to 30 minutes after infection; (ii) 0.06 μc/ml for 1-minute pulse at 30 minutes; (iii) 0.2 μc/ml for a 10-second pulse at 30 minutes. The labeled cells (about 10^{10}) were chilled rapidly, centrifuged, and resuspended in a small volume for RNA extraction as described in Fig. 1. Solid lines: infected cells. Broken lines: uninfected cells. Arrows show positions of cellular 23*S*, 16*S*, and 4*S* RNA.

could still be induced to make 27*S* RNA by infection with R17.

In Fig. 2 are shown the patterns of RNA labeling in irradiated cells, with and without infection. After a 5-minute pulse of tritiated uridine 25 to 30 minutes after infection (Fig. 2*a*), the predominant peak of radioactivity is the 27*S* viral RNA. There is also a broad peak centered at about 16*S*, and some stimulation of 4*S* labeling. After a 1-minute pulse (Fig. 2*b*), the broad 16*S* band contains more tritium than the 27*S*. On reducing the pulse time to 10 seconds (Fig. 2*c*), the label was found only in the broad 16*S* band, together with some in the 8*S* region, which probably reflects the residual background synthesis occurring in uninfected cells. There was no labeled peak at 27*S*. A 10-second pulse given at 15 minutes instead of 30 minutes after infection resulted in about half as much incorporation of the label into the 16*S* broad band. These results suggest that the 16S material, which is labeled first, may be a precursor of the 27*S* viral RNA.

The sucrose-gradient fractions containing the broad 16*S* band extracted from infected irradiated cells that received a 10-second pulse of the label were pooled, and their RNA was precipitated with ethanol, treated with ribonuclease at various concentrations, and then applied to fresh gradients (Fig. 3). Approximately 30 percent of the label and all the optical density were degraded by as little as 0.001 μg of ribonuclease per milliliter, but a resistant peak of radioactivity, sedimenting at about 12*S*, remained. Increasing the ribonuclease concentration to 0.01 μg/ml had little further effect, but at a concentration of 5 μg/ml the sedimentation rate of the resistant peak was reduced to about 8*S*. In another experiment the leading and trailing halves of the broad 16*S* band of labeled RNA were collected separately and applied to fresh gradients, with or without treatment with ribonuclease (0.1 μg/ml). The leading half of the band still sedimented ahead of the trailing half, such behavior indicating a true heterogeneity of molecular size in the original band; yet the two halves yielded indistinguishable ribonuclease-resistant peaks, and both contained 30 percent of ribonuclease-sensitive label. The labeled material appearing in the 8*S* region after a 10-second pulse of tritiated uridine was also isolated separately and found completely sensitive to ribonuclease.

These experiments show that the ribonuclease-resistant material extracted from infected cells sediments in the 16*S* region, and further suggest that the broad labeled band at 16*S* consists of a ribonuclease-resistant core with ribonuclease-sensitive tails of different lengths. After removal of the tails, the core sediments at 12*S*, which is commensurate with its being a double-stranded version of the 27*S* viral RNA, on the assumption that the double-stranded version has a rigid DNA-like structure (*11*). The core may be tentatively visualized as a double-stranded structure with the tails being incomplete new strands attached to it. The alternative possibility that the radioactivity at 16*S* consisted of 12*S* molecules aggregated with 16*S* ribosomal RNA is unlikely, since its position relative to the 16*S* ribosomal RNA was unchanged when it was dissolved in 0.01*M* EDTA (ethylenediaminetetraacetic acid) and sedimented through a

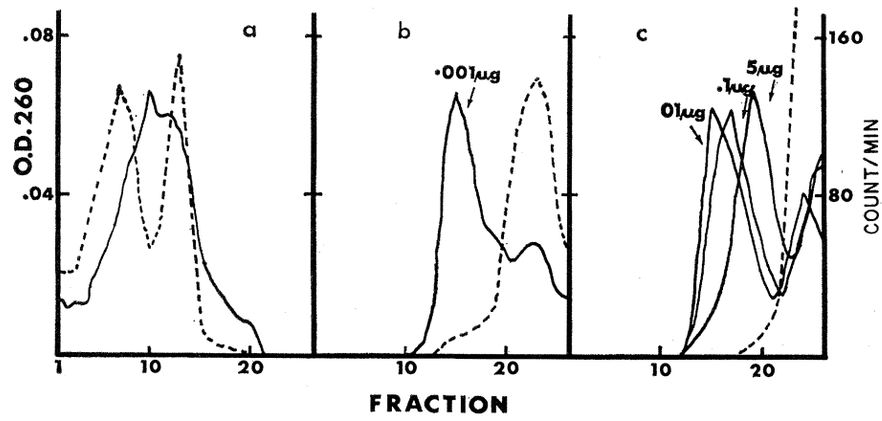

Fig. 3. Effect of ribonuclease on 10-second pulse labeled 16*S* RNA from infected irradiated cells: (i) untreated; (ii) 0.001 μg of ribonuclease per milliliter, 15 minutes at 37°C; (iii) 0.01, 0.1, 5.0 μg of ribonuclease per milliliter. Solid lines: radioactivity. Broken lines: optical density.

gradient containing 0.1*M* NaCl and 0.01*M* EDTA instead of Mg^{++}.

Attempts to obtain 27*S* single-stranded RNA from the 16*S* material by separating its supposed strands have been unsuccessful. Heating the 16*S* material to 40°C in formamide, followed by reprecipitation and centrifugation, left the 16*S* band unchanged. Nor did heating for 5 minutes at 100°C in 0.001*M* EDTA and rapid cooling affect the sedimentation rate of the 16*S* material.

Deoxyribonuclease had no effect on the 16*S* labeled material, but snake-venom diesterase (20 μg/ml, 15 minutes at 37°C, *p*H 8.5) degraded it to nonsedimenting pieces. This action may have been due to attack by endonuclease rather than exonuclease (*12*).

In order to study the fate of the labeled RNA in the broad 16*S* band, a 10-second pulse of tritiated uridine was followed by a 10-minute "chase" period in unlabeled medium. The results of gradient analysis are shown in Fig. 4*a*. During the chase, a new labeled peak of 27*S* viral RNA appeared, and there was also a large increase in 4*S* label, but the broad 16*S* region remained unchanged. However, in the ribonuclease-treated samples (Fig. 4*b*) there was a marked decline of ribonuclease-resistant label to 10 to 15 percent of the original amount during the 10-minute chase, so that by the end of the chase most of the tritium in the 16*S* region had become ribonuclease-sensitive.

The loss of ribonuclease-resistant label is approximately equal to the gain of 27*S* label during the chase. That ribonuclease-sensitive labeled RNA sedimenting at 16*S* persists during the chase may be due to recycling of some of the liberated 27*S* single strands into double-stranded form, with subsequent displacement into tails; and due also to incomplete cessation of RNA labeling during the chase (the increase of 4*S* label suggests this). Therefore a precursor-product relation between 16*S* and 27*S* RNA is suggested, but not established.

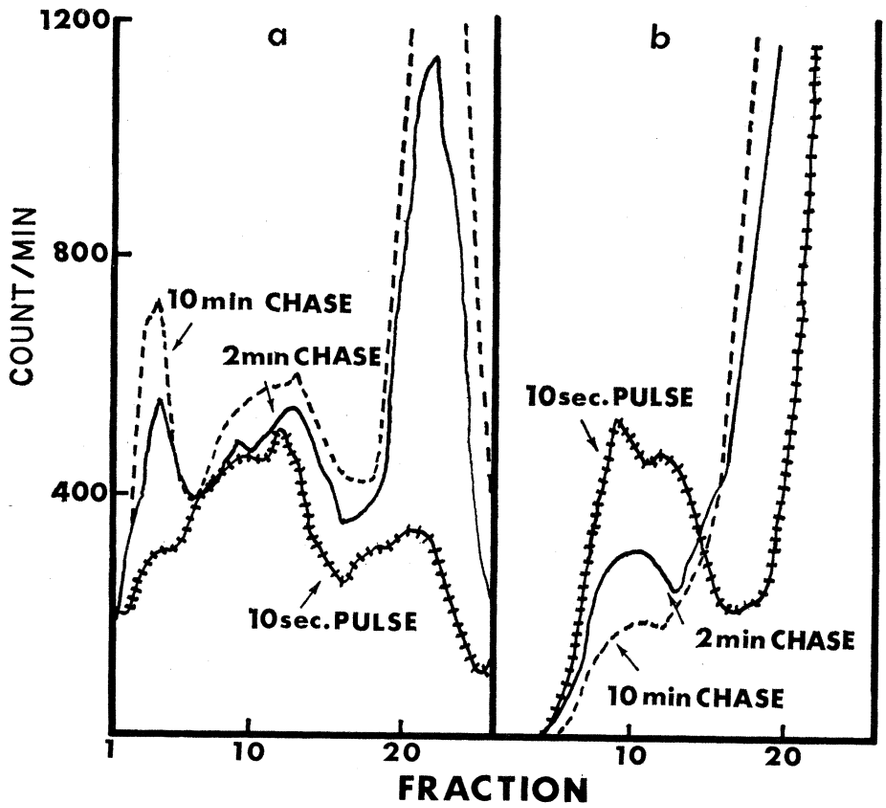

Fig. 4. Fate of pulse-labeled RNA during a chase. Cells were irradiated, infected, and labeled with a 10-second pulse of tritiated uridine 30 minutes after infection, as described for Fig. 2. They were then chilled and centrifuged; part was treated with phenol and part was suspended in nonradioactive medium containing 40 μg of uridine per milliliter for a chase period at 37°C. After 2 minutes and 10 minutes, samples were chilled and centrifuged, and their RNA was extracted as in Fig. 1. Half of each RNA sample was treated with ribonuclease (0.1 μg/ml, 15 minutes, 37°C) before gradient analysis. (i) Untreated RNA, centrifuged 4 hours at 37,500 rev/min; (ii) ribonuclease treated RNA, centrifuged 5.75 hours at 39,000 rev/min.

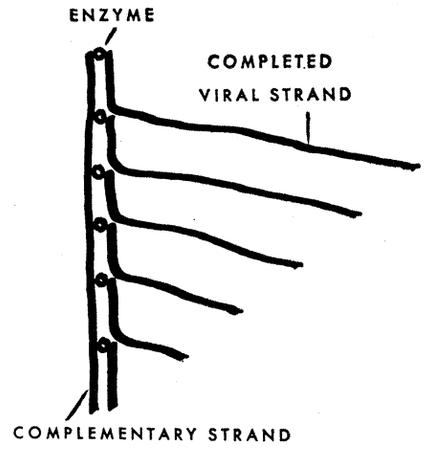

Fig. 5. Viral RNA being synthesized.

Finally, the stability of the ribonuclease-resistant part of the radioactive RNA after a 15-minute pulse of tritiated uridine (20 to 35 minutes after infection) was examined during a chase period. In this experiment, in which both strands of the RNA duplex were probably labeled, there was again a marked decline in ribonuclease-resistant label, although by the end of a 15-minute chase 25 to 35 percent of the initial label remained. Therefore, of the two strands in the ribonuclease-resistant core, one is turning over while the other may be stable.

Our results are in agreement with the hypothesis of Weissman *et al.* (*13*). According to this hypothesis a virus-induced RNA polymerase acts on a double-stranded RNA to produce new single strands of viral RNA by displacement of one of the strands, in the same way that a DNA-RNA hybrid yields single RNA strands in vitro (*14, 15*). It seems likely that the heterogeneous 16*S* RNA, which is the first species to be labeled in infected cells, may consist of strands complementary to the viral RNA, with a number of partially displaced single strands (ribonuclease-sensitive tails) hydrogen-bonded to the complementary strand over a certain region (ribonuclease-resistant core) (Fig. 5). Since viral RNA may continuously produce complementary strands and initiate new replicative-intermediate structures of the type shown in Fig. 5, there will be a population of these forms having a distribution of numbers of displaced single strands. In accordance with this view, a short pulse of radioactive uridine labels predominantly the double-

stranded part, and the label is lost during a chase, with the simultaneous appearance of labeled 27*S* RNA. After at 15-minute labeling period most (60 to 80 percent) of the 16*S* RNA is ribonuclease-sensitive.

The minimum time for labeling free 27*S* RNA molecules, which is probably about 0.5 minute in our system, would represent the time for synthesis of the most nearly finished molecule and its displacement by the next in line (the first and longest tail belongs to a completed molecule which will be displaced unlabeled). The RNA of the infecting virus appears to be absent in mature progeny (*16*). In order to reconcile this finding with our model (Fig. 5), in which the infecting strand forms a duplex and is then displaced (*13*), it must be assumed that the strands produced early in infection are less likely to be built into virus particles than those produced later.

M. L. FENWICK
R. L. ERIKSON
R. M. FRANKLIN
Department of Pathology, University of Colorado Medical Center, Denver

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Amino Acids: Incorporation into α- and β-Chains of Hemoglobin by Normal and Thalassemic Reticulocytes

Abstract. *After incubation of reticulocytes with radioactive amino acid, the specific activity of the β-chain of globin from the blood of patients with thalassemia was consistently lower than that of the α-chain. In subjects without this disorder, the specific activity of the β-chain was always equal to or higher than that of the α-chain.*

Study of the molecular basis of thalassemia is of considerable interest since the disease is the prototype of a genetically controlled anomaly in which there is a decreased production of a structurally normal protein. As such, this defect may provide a means of investigating some aspects of the control of the rate of protein synthesis in a mammalian system (*1, 2*).

We have recently been engaged in the measurement of the rate of uptake of isotopic amino acids into the α- and β-chains of rabbit hemoglobin synthesized in vitro by reticulocytes. After brief periods of labeling, relatively more isotopic leucine, lysine, or valine is incorporated into the β-chain than into the α-chain when correction is made for the difference in the number of amino acid residues in each chain. If the labeling period is longer than 15 minutes, there is a near equality in the specific activity of the two chains, as might be expected (*3*). When reticulocytes from patients with acute leukemia in remission or with sickle cell anemia were incubated with radioactive amino acids, a similar phenomenon was observed. However, when reticulocytes obtained from patients with thalassemia major were used, a prominent difference was noted between the specific activities of the α- and β-chains after relatively long incubation periods. The α-chain consistently incorporated more carbon-14 per amino acid residue than the β-chain did.

Red cells were obtained by centrifugation from heparinized whole blood and washed once with cold Borsook's saline (*4*). The cells were then suspended in a modified Krebs-Henseleit medium (*2*) and incubated at 37°C for 2.5 minutes. The C^{14}-labeled amino acid was then added, and incubation was continued. The red cells were separated from the medium by centrifugation at 4°C, washed four times with ten volumes of Borsook's saline, then lysed in 0.0015*M* magnesium chloride solution. Particulate material was removed by centrifugation, first at 10,000*g* for 20 minutes, then at 100,000*g* for 60 minutes. The supernatant was dialyzed against 0.01*M* sodium phosphate, *p*H 7.0, containing potassium cyanide (100 mg/liter) for at least 24 hours, and the hemoglobin was purified on either columns of carboxylmethylcellulose (2 by 10 cm) or on columns of amberlite CG-50 (2 by 60 cm), the latter being used for the separation of hemoglobins A and F. After application of up to 500 mg of hemoglobin, the carboxylmethylcellulose column was washed with phosphate buffer, *p*H 7, and the hemoglobin was eluted with 0.05*M* dibasic sodium phosphate. Columns of amberlite were equilibrated with 0.05*M* sodium phosphate, 0.01*M* potassium cyanide, *p*H 7.02, before application of up to 600 mg of hemoglobin. After the columns had been washed free of readily elutable hemoglobin at 4°C, the buffer was changed to 0.075*M* sodium phosphate at room temperature. By this procedure hemoglobin A containing less than 5 percent of hemoglobin F was eluted.

Table 1. Clinical and experimental data on α- and β-chains of hemoglobin.

Patient	Reticulocyte count (%)	Hgbn. purification*	Incubation (hr)	C^{14} isotope	Radioactivity (count min^{-1} mg^{-1}) α-chain	Radioactivity (count min^{-1} mg^{-1}) β-chain	S.A.$_\alpha$/S.A.$_{\beta+}$
			Sickle cell disease				
M.G.	8.2	CM	3½	Valine	226	359	0.63
			Acute leukemia (remission)				
J.H.	17	CM	4	Leucine	234	297	0.79
L.G.	4.9	CM	3	Valine	62	68	0.91
P.A.	8.0	CM	2	Leucine	48	49	0.98
			Thalassemia major				
N.L.	8.6	CM	3	Valine	183	98	1.9
N.L.	2.5	Amb	4	Valine	284	144	2.0
J.L.	3.4	Amb	4	Valine	464	201	2.3
P.T.	3.3	Amb	1	Valine	323	45	7.2

* CM, carboxymethylcellulose; Amb, amberlite. † S.A., specific activity.