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ARGONNE CANCER RESEARCH HOSPITAL
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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1972

ALEXANDER GOTTSCHALK, M.D.
Editor

ELISABETH F. LANZL, A.M.
Associate Editor

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THE USE OF ^{67}Ga SCANNING IN THE STAGING OF HODGKIN'S DISEASE*

By

D. A. Turner,[†] S. M. Pinsky,[‡] A. Gottschalk, P. B. Hoffer,[§]
J. E. Ultmann, and P. V. Harper

Recent advances in the management of Hodgkin's disease have resulted in the prospect of cure or long-term remission for a substantial number of patients. Since selection of an appropriate therapeutic regimen for an individual patient is dependent upon accurate knowledge of the extent of his disease, meticulous clinical staging has become of paramount importance (Table 1).^{1,2}

Table 1

HODGKIN'S DISEASE: ANN ARBOR MODIFICATION OF RYE STAGING SYSTEM (1971)*

Stage I:	Involvement of a single lymph node region (I) or of a single extralymphatic organ or site (I _E).
Stage II:	Involvement of two or more lymph node regions on the same side of the diaphragm (II), or localized involvement of extralymphatic organ or site and of one or more lymph node regions on the same side of the diaphragm (II _E). Optional recommendation: Number of node regions involved indicated by subscript (e.g., II ₃).
Stage III:	Involvement of lymph node regions on both sides of the diaphragm (III), which may also be accompanied by localized involvement of extralymphatic organ or site (III _E), or by involvement of the spleen (III _S), or both (III _{SE}).
Stage IV:	Diffuse or disseminated involvement of one or more extralymphatic organs or tissues with or without associated lymph node enlargement. The reason for classifying the patient as Stage IV should be identified further by defining site by symbols.

* In Hodgkin's disease, all patients are subclassified A or B to indicate the absence or presence, respectively, of (a) unexplained weight loss of more than 10 per cent of their body weight; (b) unexplained fever with temperatures above 38°C; (c) night sweats.

Gallium-67 citrate has been shown to concentrate in certain lymphomas and other soft-tissue tumors to a degree which makes these lesions detectable upon whole-body scintillation scanning.³⁻⁵ Systematic investigation of the use of ^{67}Ga scanning in clinical staging of Hodgkin's disease, however, has not been reported previously. This communication presents the

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first series of patients with Hodgkin's disease who have undergone gallium-67 scanning and in whom sites of tumor involvement subsequently have been proved at laparotomy. A summary of these findings has been published.⁶

MATERIALS AND METHODS

Since August 1, 1970, all previously untreated patients with biopsy-proved Hodgkin's disease seen at the University of Chicago Hospitals and Clinics have undergone whole-body scanning with ⁶⁷Ga-citrate as part of a previously established Hodgkin's disease staging protocol. Selected patients with suspected recurrent Hodgkin's disease have been evaluated similarly.

As of July 1, 1971, 20 patients who had been evaluated by ⁶⁷Ga scanning had undergone exploratory laparotomy for the purpose of clinical staging. The staging laparotomy includes multiple excisional biopsies of lymph nodes (splenic hilar, porta hepatis, mesenteric, right and left aortic, iliac, and inguinal nodes), splenectomy, wedge biopsy of the liver, needle biopsies of the right and left lobes of the liver, and core biopsy of iliac bone.^{7,8} Patients determined to be Stage IV on the basis of the prelaparotomy protocol and patients who had received chemotherapy (except prednisone) within four months preceding evaluation were excluded from the present study because they are not subjected to laparotomy. Histologic confirmation of the scan data, therefore, could not be obtained.

Scan method. Carrier-free ⁶⁷Ga-citrate was prepared in the Argonne Cancer Research Hospital's compact cyclotron (Cyclotron Corporation CS-15) by bombardment of natural zinc with 8-MeV deuterons.⁹ Seventy-two hours prior to scanning, patients received 1.5 to 3.0 mCi of ⁶⁷Ga-citrate in a volume of less than 2 ml by intravenous injection. As this study progressed, it was found that colonic activity created considerable difficulty in the interpretation of the scans. Some of the patients in this series have therefore received cleansing enemas immediately prior to scanning. Patients were scanned from head to knee with an Ohio Nuclear Model 84 radionuclide scanner fitted with dual 12.7 cm detectors and 38H collimators (9 cm focal depth). Simultaneous anterior and posterior scans were made with patients in the supine position. The upper detector was adjusted to a level 2 to 4 cm above the anterior surface of the patient's body. Scanning speeds of 650 to 750 cm/minute were used, the entire scan taking 45 to 75 minutes. A 20 per cent window was set around the 296-keV photon. Maximum information densities of 15 to 75 counts/cm² were obtained by this method. A five-to-one minification readout was utilized.

Scan interpretation. The scans were interpreted by two of the authors (A.G. and P.B.H.) without knowledge of the patient's identity, clinical history, or the result of other studies. They were evaluated for areas of abnormally high uptake in the cervical, axillary, mediastinal, para-aortic, iliac, and inguinal regions as well as in the spleen. The liver and porta hepatis cannot be evaluated with this technique since the liver image is normally dense (Figure 1).

The scan results were compared with the extent of disease as determined by pathological examination of the surgical specimens and by chest radiography. In the case of unbiopsied node masses in the cervical, axillary, and inguinal regions, enlargement documented by physical examination was taken as evidence of the presence of disease. Involved supraclavicular and adjacent cervical or axillary nodes were counted as one area of involvement, as were para-aortic and mesenteric nodes (para-aortic-mesenteric area).

Figure 1. Case 3. Normal ^{67}Ga scan. a. Anterior view. b. Posterior view. The radionuclide has accumulated principally within the liver.

RESULTS

A detailed tabulation of the results appears in Tables 2 and 3. Twenty-three of twenty-nine disease sites (79 per cent) were identified correctly by ^{67}Ga scanning. There were six "false negatives" (Cases 8, 10, 11, and 20) and six "false positives" (Cases 9, 10, 15, 18, and 20). The spleen alone accounted for three "false positive" areas (Figure 3). One "false positive" area was in the mediastinum (Case 20). This represents failure of the ^{67}Ga scan to correlate with the patient's normal chest radiograph, the appearance of which, of necessity, has been taken as the standard of mediastinal involvement in this series. Two "false negatives" were the result of failure to identify tumor activity in the iliac areas (Case 8). There was, in fact, considerable activity in the iliac regions, but this was interpreted as being due to radionuclide within the colon (Figure 4). One "false positive" occurred in the right inguinal region (Case 10). Correlation between evaluation of the axilla by physical examination and ^{67}Ga scan was poor. Two of three axillary areas which were thought on the basis of physical findings to be involved failed to be identified by the ^{67}Ga scan (Cases 8 and 11) (Figure 4). In seven patients, the ^{67}Ga scans

Figure 2. Case 5. Abnormal ^{67}Ga scan. a. Anterior view. b. Posterior view. Para-aortic-mesenteric area (arrow) and splenic involvement are well demonstrated by this study. The mediastinum was correctly read as positive although the abnormality is subtle. Compare the appearance of involved areas with similar areas in Case 3 (Figure 1).

revealed no evidence of disease: In none of these patients was disease documented at staging laparotomy, by physical examination, or by chest radiography.

Gallium-67 scanning was particularly useful in two patients being surveyed for possible recurrence of previously treated Hodgkin's disease. One individual* was admitted to the University of Chicago Hospitals and Clinics because of systemic manifestations suggestive of recurrent disease. Extensive conventional diagnostic investigation revealed no evidence of recurrence. A ^{67}Ga scan, however, showed extremely high accumulation in the left upper quadrant of the abdomen, in spite of the fact that the spleen had been removed at a previous staging laparotomy two years earlier. On the basis of the ^{67}Ga scan alone the patient underwent a second

* This case has not been included in our series because extensive lymph node biopsy was not performed at laparotomy.

Table 2

COMPARISON OF SCAN RESULTS WITH CONFIRMED SITES OF DISEASE

Case No.	Sites of involvement	⁶⁷ Ga finding
1	M	+
2	M, P-M	+, +
3	None	None
4	None	None
5	M, P-M, Sp	+, +, +
6	None	None
7	None	None
8	N-Ax, M, P-M, Il, Il, Sp, Portal	+Fn, +, +, Fn, Fn, +, U
9	N, M	+, +, false positive Sp
10	S, M	Fn, ±, false positive P-M and Ing
11	Ax, M	Fn, +
12	Chest wall, Portal	+, U
13	M	+
14	None	None
15	N	+, false positive Sp
16	None	None
17	M, Sp	+, +
18	N-Ax, M	+, +, false positive Sp
19	None	None
20	N, P-M, Sp	+, Fn, +, false positive M

Abbreviations: Fn = False negative, U = Uninterpretable area, N = Neck, S = Supra-clavicular area, M = Mediastinum, Ax = Axilla, Il = Iliac area, Ing = Inguinal area, Sp = Spleen, P-M = Para-aortic and mesenteric areas.

exploratory laparotomy, and a large tumor mass in the splenic bed was disclosed. In a second individual, pain developed in the right anterior chest wall during radiotherapy for recurrent disease elsewhere (Case 12). Physical and radiographic examinations were unrevealing. An area of high concentration of ⁶⁷Ga was demonstrated in the region of discomfort (Figure 5). Surgical biopsy revealed a focus of Hodgkin's disease between the ribs, for which additional radiotherapy was administered.

DISCUSSION

Gallium-67 citrate was first advocated as a tumor-scanning agent by Edwards and Hayes, who noted fortuitous localization of the radionuclide in soft-tissue tumor while investigating its use in bone scanning.⁴

The physical properties of ⁶⁷Ga are acceptable for radionuclide scanning in humans. It has a half-life of 78 hours, decaying by electron capture with the following discrete photon emissions: 93 keV (40 per cent), 184 keV (24 per cent), 296 keV (22 per cent), and 388 keV (7 per cent).¹⁰

Table 3
SUMMARY OF COMPARISON OF ^{67}Ga SCAN RESULTS
WITH CONFIRMED SITES OF DISEASE

Involved sites	Neck, supra-clavicular area, and axilla	Mediastinum	Para-aortic and mesenteric areas	Iliac area	Inguinal area	Spleen	Other areas	Total
Involved sites	8	10	4	2	0	4	1*	29
Sites correctly identified	5	10	3	0	0	4	1*	23
False negatives	3	0	1	2	0	0	-	6
False positives	0	1	1	0	1	3	0	6

* Chest wall

Figure 3. Case 9. Abnormal ^{67}Ga scan. a. Anterior view. b. Posterior view. Abnormally high activity is seen in the right neck, mediastinum, and spleen. Pathological examination demonstrated a normal spleen, making this a "false positive." Compare with splenic activity in Figure 1. Also note activity in the colon (3, a). Compare marrow and bone uptake in the pelvis with that of Case 3 (Figure 1, b). Bone and marrow uptake varies greatly from patient to patient.

These energies are suitable for imaging with scintillation cameras as well as with rectilinear scanning devices.⁴ The total-body radiation dose from ^{67}Ga is estimated to be 0.34 rads/mCi.⁴

When carrier-free ^{67}Ga -citrate is injected intravenously, it is bound to transferrin and other plasma proteins.¹² It leaves the blood slowly and accumulates in kidney, liver, spleen, lung, marrow, and bone.^{11,12} Maximum tumor-to-background activity ratios are obtained in humans 48 hours or more after injection.⁵

Workers at Oak Ridge,^{13,14} and others,¹¹ have recently directed their attention toward elucidation of the mechanisms by which ^{67}Ga is concentrated by soft-tissue tumors. These mechanisms are not yet well understood. Although viable tumor cells in experimental animals have been shown to accumulate the tracer, considerably greater radionuclide concentration has been found associated with macrophages scattered within the tumor as well as within reticulo-

Figure 4. Case 8. Abnormal ^{67}Ga scan. a. Anterior view. b. Posterior view. Note prominent colonic activity. Areas of high activity are seen in both iliac fossae. These were interpreted as being due to activity within the cecum and sigmoid colon. At surgery, however, lymphoma was found in both iliac areas. Activity in the neck, mediastinum, para-aortic-mesenteric areas and the spleen were correctly interpreted as being due to the presence of lymphoma. Disease in the left axilla was missed. An involved portal node could not be identified because of overlying liver activity.

endothelial cells of the liver and spleen.¹⁴ Gallium-67 seems to be bound to a macromolecular agent or agents located within an intracellular granule tentatively identified as a lysosome-like organelle.¹³

The ability to accumulate ^{67}Ga -citrate is not limited to neoplastic tissue and the various normal tissues listed above. The agent has been shown to be concentrated at sites of inflammation as well.¹¹ It also has been reported to be concentrated by the breast of a patient with gynecomastia.⁵ Accumulation by the breasts was noted in one of our female patients scanned shortly after termination of pregnancy.

In spite of the apparent nonspecificity of ^{67}Ga uptake, our experience with gallium scanning in Hodgkin's disease has been encouraging. Our success has been augmented by the fact that

Figure 5. Case 12. Anterior view. An area of right anterior chest wall involvement is clearly seen in spite of underlying liver activity. Note high activity in nasopharyngeal area. Gallium-67 uptake in this area is variable and does not correlate with the presence of lymphoma in the pharynx.

patients in our series, with one exception, lacked intercurrent inflammatory disease, eliminating that source of "false positive" scans. The automatic exclusion of patients having received chemotherapy within the four months preceding evaluation at our institution may also have been a factor, since effective chemotherapy or radiotherapy greatly reduces radionuclide concentration in treated areas.¹⁵

The propensity of certain normal tissues to concentrate ^{67}Ga does, of course, create problems. As indicated above, normal accumulation by the liver precluded detection of disease in the porta hepatis (Cases 8 and 12). Interpretation of the spleen also presents a difficult problem. Although we correctly identified all four involved spleens in this series, three of six "false positives" were due to accumulation of relatively high levels of radionuclide by normal spleens (Figure 3). Activity within the colon also has been troublesome and may have accounted for two of our "false negatives" (Figure 4). We hope that more vigorous bowel preparation (60 ml castor oil on the day prior to scanning) will reduce the latter problem.

We have recently introduced another modification of our technique which may significantly improve our results. Heretofore we have used only the 296-keV photon emission for scanning. Our scanning instrument has been modified, so that we are now able to use all three major photon emissions (93, 184, and 296 keV) simultaneously. Although few scans have been completed with this new system, preliminary results are promising. Maximum information densities more than four times that obtained using the 296-keV emission alone have been achieved without changes in the scanning speeds. Considered subjectively, spatial resolution of the three emission scans has been significantly better than that of the single-emission (296 keV) scans. Whether or not improved spatial resolution will be translated into improved diagnostic accuracy remains to be seen.

Finally, we believe ^{67}Ga scanning is a useful supplement in the staging of Hodgkin's disease. In certain patients it may be the only noninvasive study that will correctly identify sites of tumor.

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ASPECTS OF IMAGING AND COUNTING IN NUCLEAR MEDICINE USING SCINTILLATION AND SEMICONDUCTOR DETECTORS*

By

R. N. Beck, L. T. Zimmer, D. B. Charleston, and P. B. Hoffer

In the interest of minimizing patient irradiation, while maximizing diagnostic image quality, it is desirable to utilize all of the radiation emerging from the patient. For optimum utilization, it appears to be necessary to allow photons with different energies to contribute to the image with different weights. Optimum weights have been determined for the most common case of noise-limited images, where it is assumed that the weights should be chosen so as to maximize the signal-to-noise ratio. For less noisy images, some signal-to-noise ratio may be sacrificed. In this case, image sharpening may be achieved by assigning negative weights to scattered photons; that is, by scatter subtraction. In general, image formation with multiple weighted channels provides a greater degree of flexibility than is possible with a single channel.

The principal goal of research on instrumentation in nuclear medicine is to improve the diagnostic quality of information derived from imaging procedures. These procedures include imaging of static and dynamic in vivo distributions of suitable stable and radioactive tracers. To minimize patient irradiation, while maximizing diagnostic image quality, it is necessary to make optimum use of the radiation emerging from the patient, a significant fraction of which consists of scattered photons due to Compton interactions within the patient. The question how best to treat scattered radiation is a subject of considerable interest, since scatter affects both noise and contrast in the image. These effects are directly related to the effects of scattered radiation on the sensitivity and spatial resolution of the imaging system. This paper summarizes the current status of our work on various aspects of the problem, and suggests possible directions for the future.

PREVIOUS CONSIDERATIONS AND RESULTS

The object to be imaged. If attention is confined to static radionuclide imaging, the object to be imaged can be thought of as a low-intensity, self-radiant, three-dimensional distribution of radioactive material embedded in a turbid medium which scatters and absorbs primary radiation emitted by the object. Thus, the radiation emerging from the patient consists of a line spectrum of unscattered photons and a continuous spectrum of photons scattered at all angles, which may be due to γ rays emitted outside the detector field of view, as indicated on the left in Figure 1.

The radiation detector. Since the energy resolution of radiation detectors is imperfect, the pulse amplitude spectra due to scattered and unscattered photons overlap; thus, it is impossible to reject all pulses due to scattered photons by pulse-height analysis without rejecting the pulses due to primary radiation as well. A method for separating these components in the region

* This report is taken from a paper² that appeared in IEEE Transactions on Nuclear Science, NS-19(3): 173, 1972.

Figure 1. In brain scanning with ^{99m}Tc and a NaI(Tl) detector, the output pulse amplitude spectra due to scattered and unscattered photons overlap appreciably because of imperfect energy resolution of the detector.

of overlap has been developed¹ for analyzing spectra from large volume distributions. The result for ^{99m}Tc , using a NaI(Tl) scintillation detector, is shown in Figure 1, lower right.

The scatter fraction, f_s : effect on sensitivity and dependence on E_B and θ . The separation of these spectra enables us to compute the scatter fraction, f_s^* which is defined as the fractional increase in response due to scattered photons over the response to properly collimated primary

*To conform with the notation used in reference 2, the symbol f_s will be used here in place of S , which was used in references 1, 3, and 4.

radiation. Thus, one effect of scatter is an increase in response (or apparent sensitivity of the detector) by the factor $(1 + f_s)$. This has the effect of reducing the relative magnitude of random fluctuations or quantum noise in the image.

Clearly, the magnitude of the scatter fraction is a function of E_B , the base line setting of the single-channel pulse height analyzer (SCA) [the upper discriminator is set just above the unscattered photopeak]. For a given primary photon energy, E_O , the expected value of the maximum scattering angle, θ , for accepted photons is determined by E_B from Compton's equation. As E_B is raised, the angle of acceptance is reduced. As a result, f_s and sensitivity are reduced, as shown in Figure 2. Here f_s is plotted as a function of θ for four γ -ray energies, for spectra

Figure 2. When the scatter fraction f_s is plotted as a function of the photon scattering angle corresponding to the angle of acceptance of the base-line setting, values of f_s are similar for all isotopes considered. The dashed lines indicate the "optimum" settings, assuming that scatter is like background.

obtained from uniform volume distributions 16 cm in diameter and 16 cm high in water, simulating the patient's head. Since f_s depends on the probability that Compton scattering occurs within the patient, as well as the probability that the scattered photon will emerge to be detected, it is of interest to note in Figure 2 that f_s is not radically different for primary radiation in the range from 140-510 keV, for values of E_B corresponding to the same angle of acceptance. We might, therefore, expect to observe approximately the same effects of scatter on image contrast and resolution for these radionuclides when the lower discriminator is set for the same angle of acceptance.

First criterion for optimum treatment of scattered radiation. Since scattered photons may be due to primary radiation emitted at any point within the source, it is reasonable to assume as a first approximation that the scattered photons produce essentially the same effect as back-

ground, contribute more or less uniformly to all parts of the image, and reduce image contrast by the factor $1/(1 + f_s)$. In that case, the base line setting which minimizes the error in measuring the count rate due to unscattered photons, in the presence of a "background" due to scatter, may be regarded as the optimum setting. This error is given by

$$\epsilon = \sqrt{\frac{1 + f_s}{N_p \Psi}}, \quad (1)$$

where N_p is the total number of pulses due to primary photons within the photopeak, and Ψ is the fraction of these within the SCA window. Thus, Ψ and f_s are functions of E_B . On this assumption, the optimum base line settings for the sources considered in Figure 2 are given approximately by 0.9 times the primary γ -ray energy, E_0 . For these settings, less than 5 per cent of the pulses due to unscattered photons are rejected.³ The dashed lines in Figure 2 indicate the scatter fractions and acceptance angles corresponding to these optimum settings. The associated scatter fractions differ by more than a factor of 2, and increase monotonically with decreasing γ -ray energy, with a value of $f_s = 0.38$ for ^{99m}Tc .

For a given value of N_p , the error ϵ can be further reduced only by reducing f_s and/or by increasing Ψ . This can be achieved by use of a detector with better energy resolution.

Use of a large Ge(Li) detector. Figure 3 shows the pulse amplitude spectra obtained from a brain phantom using ^{99m}Tc with a NaI(Tl) detector and a large coaxial Ge(Li) detector, the latter having an area of approximately 14 cm^2 and a length of approximately 6 cm. The values of the full width at half maximum (FWHM) are 23 keV and 4 keV, respectively, for these detec-

Figure 3. Improved energy resolution obtainable with a Ge(Li) detector permits rejection of all but small-angle scatter. The angle of acceptance is 55° for $E_B = 125 \text{ keV}$ and 25° for $E_B = 136 \text{ keV}$.

tors. Although the Ge(Li) detector is not a high-resolution detector by the standards of research in physics, it nevertheless permits the lower discriminator to be raised to 136 keV before any pulses due to unscattered photons are rejected. For this setting the angle of acceptance is 25° .

The effect of this on brain scans is shown in Figure 4. The image on the left was obtained with two NaI(Tl) detectors operated simultaneously to reduce scanning time. The image on the right was obtained with the Ge(Li) detector. The increase in image contrast over the brain lesion has been verified by sensitometric measurements on the film.

Figure 4. Lateral brain scans of the same patient with NaI(Tl) detectors (left) and a Ge(Li) detector (right). Note the increased image contrast and improved perceptibility of the lesion.

Although increased rejection of scatter clearly results in improved image contrast, the principal problem with this Ge(Li) detector is reduced sensitivity; that is, we observe only about one third as many counts as with a 2"-diameter NaI(Tl) crystal, for which the collimator used in both scans was designed.

As a result of reduced sensitivity, the scanning time with the Ge(Li) detector is excessive, being approximately 45 minutes for one view of the head. Such a detector is not truly competitive with the standard 3" NaI(Tl) detectors commonly used. In fact, an almost identical result could be obtained (in terms of increased contrast and reduced sensitivity) by operating the scintillation detector with a base-line setting of 146 keV, well above the optimum setting of 125 keV for this detector.⁴

To some extent, this limitation will ultimately be overcome by 7.5 cm-diameter Ge(Li) detectors with a drifted depth greater than 1 cm, and FWHM < 4 keV, which are believed to be feasible.

Meanwhile, we have pursued the question how best to utilize information obtained from Ge(Li) detectors.

Second criterion. The assumption that scatter is like background is reasonably valid for low-energy γ rays, using a scintillation detector, where the angle of acceptance corresponding to the optimum base-line setting is large. However, this assumption is less valid for high-energy γ rays, using a NaI(Tl) detector, and for low-energy γ rays, using a Ge(Li) detector, since only photons scattered through small angles are accepted. In these cases, image contrast is degraded less by scatter, since photons scattered through small angles tend to resolve object structures, to a degree that depends on the structure size.

In these situations, where scattered radiation tends to resolve object structures of interest, a more general and appropriate criterion for optimizing the base-line setting is the figure of merit,

$$Q = \frac{(C_t - C_o)^2}{(C_t + C_o)}, \quad (2)$$

where C_t is the count rate over a suspected tumor and C_o is the count rate over normal tissue. Scattered photons may increase Q only if the scatter components of C_t and C_o are different, i.e., only if the image contrast due to scattered photons is non-zero. Maximizing this quantity is equivalent to maximizing the signal-to-noise ratio that can be achieved per unit of observation time to distinguish between C_t and C_o .

Although some limited use has been made of this criterion to optimize the base-line setting E_B for scintillation detectors,⁴⁻⁶ it serves here as the basis for a more general formulation, discussed below under Fourth Criterion.

AN EXTENSION AND GENERALIZATION

Scatter subtraction. Heretofore we have considered only the possibilities of accepting or rejecting pulses due to scattered photons. An analysis by Zimmer suggests that subtraction of scatter may provide an attractive alternative, since this would produce a sharpened image. This alternative has been explored superficially in the following experiment.

Figure 5 shows two SCA windows set on a ^{99m}Tc spectrum obtained with the Ge(Li) detector. The upper window brackets the primary peak, while the lower window accepts pulses due to photons scattered through angles between 25° and 55° .

Figure 6 shows the response of this system to a line source of ^{99m}Tc in a scattering medium, while Figure 7 shows the result of adding and subtracting these response functions. Addition yields a response function similar to that ordinarily obtained with a scintillation detector and a single window, while subtraction yields a bipolar function with a positive central region and negative side lobes. The latter is the typical shape of a filter function that produces a sharpening effect.

Figure 8 shows the expected images of a step source, obtained by convoluting these response functions with a unit step function. The images have been normalized to show the effect of the response functions on the shape of the image. The least sharp image was obtained with the scatter channel alone, while the sharpest image was obtained by subtracting the scatter channel from the peak channel.

This result is consistent with expectations based on a comparison of the modulation transfer functions (MTF) associated with these response functions, as shown in Figure 9. Since the

Figure 5. The peak due to unscattered photons is bracketed by one SCA, while another covers the range of photons scattered through angles between 25° and 55° .

Figure 6. Response functions for a line source in a scattering medium for the SCA windows shown in Figure 5. Note long tails due to Compton scattering within this medium.

Figure 7. The sum and difference of response functions in Figure 6. Note the bipolar nature of Peak-Scatter.

Figure 8. Expected images of a unit step obtained with response functions in Figures 6 and 7. Note that scatter subtraction yields the sharpest image.

Figure 9. Modulation Transfer Functions for the response functions in Figures 6 and 7. Note that scatter subtraction yields uniformly highest MTF.

$MTF(\nu)$ is a measure of the efficiency with which modulation of object sinusoids is transferred to the image, an ideal imaging system would have an $MTF(\nu) = 1$ for all spatial frequency components in the object. In Figure 9, the transfer function associated with scatter subtraction rises slightly above 1 in the low-frequency range and is uniformly highest at all spatial frequencies.

The overshoot observed with scatter subtraction in Figure 8, and values of $MTF > 1$ in Figure 9, indicate that too much scatter has been subtracted in this instance. That is, if less scatter were subtracted, or if less weight were given to pulses in the scatter channel, we might obtain an image without overshoot, which would look more like the object, except for the residual unsharpness due essentially to the collimator alone. This fact suggests a more general formulation of the problem of the best way of treating scatter.

Continuous weights. In the preceding discussion we have considered the consequences of accepting, rejecting, and subtracting scattered radiation in the image-forming process. These alternatives can be described as a choice between weighting factors of +1, 0, and -1 for scattered photons.

Clearly, this does not exhaust the possibilities, since we can easily conceive of imaging processes in which any finite positive or negative weight is given to each detected photon. In any particular case, the weight might be a function of the photon energy, the absolute or relative number of photons at each energy, position or time, etc., or any combination of such parameters.

Optimum weights. In general, if we conceive of the situation as one in which a human observer-analyst is called upon to extract diagnostic information from images, then each detected photon should be given a weight that depends upon the degree to which photons in its class (however defined) contribute to the formation of an image in which structures relevant to an accurate diagnosis will be detected by the observer-analyst with greatest probability.

In particular, if attention is confined to photon energy as a parameter, and if the entire pulse amplitude spectrum is divided into k discrete channels with width ΔE , and a fixed weight w_j ($j = 1, 2, \dots, k$) is given to pulses in each channel, the question that arises is: "What is the optimum weight for each channel?"

In general, the answer to this question depends upon the criterion adopted, which in turn depends upon a variety of factors concerning the objects to be imaged, the imaging system, and the observation time. Only two special cases will be discussed briefly.

Third criterion (applicable to the "noise-free" image). If the number of photons recorded per unit area in the image is so large that the signal-to-noise ratio is high even for the smallest structures of interest, then weighting factors may be chosen to maximize the correspondence between the object and image. (Linfoot⁷ has discussed several measures of correspondence that may be useful.) This will in general require sharpening the image to compensate for imperfect spatial resolution of the collimated detector. All such sharpening procedures reduce the signal-to-noise ratio, and image sharpening by scatter subtraction is no exception; however, there appears to be no a priori reason to believe that this method would be more costly of signal-to-noise ratio than existing methods.

Fourth criterion (applicable to the "noise-limited" image). The most common situation is also the most difficult; namely, when the signal-to-noise ratio is so low that every effort must be made to raise the signal above the threshold of detectability.

If we wish to maximize the signal-to-noise ratio for a particular spatial frequency component, ν (cycles/cm), it is shown in Appendix B [eq. A(12)] that the weight which should be given to pulses in the j th channel is simply proportional to the value of the Detector Transfer Function* $D'_j(\nu)$ at the frequency ν , for that channel. For the particular case discussed above, the optimum weights for the Peak and Scatter channels can be read from the respective $MTF(\nu)$ graphs in Figure 9.

In addition, if we wish to maximize the signal-to-noise ratio, SNR, for detecting an arbitrary object structure, it is shown in Appendix C that the optimum weight for each channel is simply proportional to the image contrast provided by the channel. The resulting maximum rate at which $[SNR]^2$ can be achieved is equal to the sum of figure-of-merit values for all channels. [See eq. A(19).]

DISCUSSION AND CONCLUSIONS

Implementation of variable weights. The theory of variable weights provides an extremely general basis on which to develop a theory of optimum utilization of all radiation that emerges from the patient.

In the absence of precise knowledge about the values of weighting factors, $w(E)$, that would maximize the probability of an accurate diagnosis, the most conservative approach would be to record the amplitude, as well as the position coordinates, of each detected photon for subsequent off-line synthesis and examination of multiple images, based on different assumptions concerning optimum weights. These assumptions might include the entire range of weights between the

* This function is the Fourier Transform of the detector spread function, and has heretofore been called the Modulation Transfer Function in nuclear medicine.¹⁻⁶ To conform with nomenclature in optics and radiology, it is suggested⁸ that the latter term be used only to refer to the absolute value of $D'(\nu)$; that is, $MTF(\nu) \equiv |D'(\nu)|$. When $D'(\nu)$ is real and positive, $MTF(\nu) = D'(\nu)$.

extremes implied by the criteria for the noise-free and noise-limited images described above.

If stable optimum weights should be found for routine, standardized imaging procedures, it might be desirable to implement these weights on-line, to eliminate further image processing. Although any number of techniques can be imagined for accomplishing this end, the design of a practical system may provide a stimulating challenge.

In view of the superior energy resolution of Ge(Li) and Si(Li) semiconductor detectors over NaI(Tl) scintillation detectors, any such variable weighting procedure might best be accomplished with a semiconductor detector. However, the sensitivity of semiconductor detectors must be increased before they become truly competitive with scintillation detectors.

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APPENDICES

A. The Detector Transfer Function, $D(\nu)$

Assume that the energy range of the pulse amplitude spectrum is divided into k channels with width ΔE . Let $L(x; E_j) \equiv L_j(x)$ be the detector line source response function for the j th channel, and let

$$n_j = \int_{-\infty}^{\infty} L_j(x) dx \quad A(1)$$

be the number of counts expected to occur in this channel per unit observation time. If w_j is the weight given to each of these counts, the overall weighted line source response function is given by

$$L_D(x) = \sum_{j=1}^k w_j L_j(x) = \sum_{j=1}^k w_j n_j \ell_j(x), \quad A(2)$$

where $\ell_j(x)$ is the line spread function for the j th channel, and

$$\int_{-\infty}^{\infty} \ell_j(x) dx = 1 \text{ for all } j. \quad A(3)$$

With Σ designating $\sum_{j=1}^k$, the Detector Transfer Function $D'(\nu)$ is defined by

$$D'(\nu) \equiv \frac{\text{F.T.}[L_D(x)]}{\int_{-\infty}^{\infty} L_D(x) dx} = \frac{\Sigma w_j n_j D'_j(\nu)}{\Sigma w_j n_j}, \quad A(4)$$

where the Detector Transfer Function for the j th channel is

$$D'_j(\nu) \equiv \text{F.T.}[\ell_j(x)] = \int_{-\infty}^{\infty} \ell_j(x) e^{-i2\pi\nu x} dx = \frac{\int_{-\infty}^{\infty} L_j(x) e^{-i2\pi\nu x} dx}{\int_{-\infty}^{\infty} L_j(x) dx}. \quad A(5)$$

In this nomenclature,⁸ the overall Modulation Transfer Function for the detector is defined by

$$\text{MTF}_D(\nu) \equiv |D'(\nu)|, \quad \text{A(6)}$$

and for the j th channel, by

$$\text{MTF}_j(\nu) \equiv |D'_j(\nu)|. \quad \text{A(7)}$$

B. Optimum Weighting Factors for Detection of an Object Sinusoid

Let $N_j(x)$ [$\frac{\text{counts}}{\text{cm}^2}$] define the image of an object sinusoid with modulation $m_o(\nu)$, mean intensity $\bar{o}$ [$\frac{\text{photons}}{\text{cm}^2\text{-sec}}$], and spatial frequency ν [cycles/cm], formed by the j th channel; thus,

$$N_j(x) = \bar{N}_j + \tilde{N}_j(\nu) \cos 2\pi\nu x. \quad \text{A(8)}$$

The image modulation is given by

$$m_{Ij}(\nu) \equiv \frac{\tilde{N}_j(\nu)}{\bar{N}_j} = m_o(\nu) D'_j(\nu). \quad \text{A(9)}$$

If the signal associated with this sinusoid is regarded as the difference in the expected numbers of counts under positive and negative half-cycles, then the signal for the j th channel is proportional to $\tilde{N}_j(\nu)$.

Similarly, if the noise associated with this sinusoid is regarded as the random fluctuation about the signal, measured by the standard deviation about the signal, then the noise associated with the j th channel is proportional to $\sqrt{\bar{N}_j}$.

If each count in the j th channel contributes to the image with weight w_j , the total weighted signal for all channels is proportional to $\sum w_j \tilde{N}_j(\nu)$, the total weighted noise is proportional to $\sqrt{\sum w_j^2 \bar{N}_j}$, and the [signal-to-noise ratio]² is given by

$$[\text{SNR}]^2 \propto \frac{[\sum w_j \tilde{N}_j(\nu)]^2}{\sum w_j^2 \bar{N}_j}. \quad \text{A(10)}$$

The optimum weight, w_j , which maximizes SNR, is found by solving $\frac{d[\text{SNR}]^2}{dw_j} = 0$ for w_j . Using eq. (9), this procedure yields

$$w_j = D'_j(\nu) \frac{\sum w_j^2 \bar{N}_j}{\sum w_j D'_j(\nu) \bar{N}_j}, \quad \text{A(11)}$$

which is satisfied when

$$w_j(\text{opt}) = D'_j(\nu). \quad \text{A(12)}$$

With optimum weights, and using eq. (9), the maximum value of $(\text{SNR})^2$ is

$$[\text{SNR}]^2_{\text{MAX}}(\nu) \propto m_o^2(\nu) \sum \bar{N}_j [D'_j(\nu)]^2 = \bar{o} m_o^2(\nu) \sum_{j=1}^k \bar{S}_j [D'_j(\nu)]^2, \quad \text{A(13)}$$

where

$$\bar{S}_j \equiv \frac{\bar{N}_j}{\sigma} \quad A(14)$$

is the sensitivity of the j th channel to a uniform plane distribution.

Similarly, the Detector Transfer Function with optimum weights is found by substituting eq. (12) in eq. (4), and, recognizing that $n_j \propto S_j$,

$$D'(\nu)_{\text{opt}} = \frac{\sum n_j [D'_j(\nu)]^2}{\sum n_j [D'_j(\nu)]} = \frac{\sum \bar{S}_j [D'_j(\nu)]^2}{\sum \bar{S}_j [D'_j(\nu)]} \quad A(15)$$

Equation (13) is a generalization of the result obtained previously for a single channel.³

C. Optimum Weighting Factors for Detection of Arbitrary Objects

Let N_{tj} and N_{oj} be the expected numbers of counts in the j th channel associated with a suspected tumor region, and a normal region of the same size, respectively. Thus, if the area of the tumor region is A_t , then

$$N_{tj} \equiv \iint_{A_t} N_j(x,y) dx dy,$$

and

$$N_{oj} \equiv \iint_{A_o} N_j(x,y) dx dy.$$

Let $(N_{tj} - N_{oj})$ be the signal associated with this channel and let the standard deviation about the signal, $\sqrt{N_{tj} + N_{oj}}$, be a measure of the noise.

The total weighted signal and noise for all channels are given by $\sum w_j (N_{tj} - N_{oj})$ and $\sqrt{\sum w_j^2 (N_{tj} + N_{oj})}$, respectively. The [signal-to-noise ratio]² is given by

$$[\text{SNR}]^2 = \frac{[\sum w_j (N_{tj} - N_{oj})]^2}{\sum w_j^2 (N_{tj} + N_{oj})} \quad A(16)$$

Solving $\frac{d[\text{SNR}]^2}{dw_j} = 0$ for w_j yields the optimum weight,

$$w_j(\text{opt}) = \frac{(N_{tj} - N_{oj})}{(N_{tj} + N_{oj})}, \quad A(17)$$

which can be interpreted as a measure of the contrast provided by the j th channel. In general, the contrast, and therefore the optimum weight, will depend on the size of the suspected tumor region.

Using optimum weights,

$$[\text{SNR}]_{\text{MAX}}^2 = \sum_j \frac{(N_{tj} - N_{oj})^2}{(N_{tj} + N_{oj})} \quad A(18)$$

If t (sec) is the observation time for each of these regions, which produce mean count rates

C_{tj} and C_{oj} , respectively, then the maximum rate at which $[\text{SNR}]^2$ can be achieved is given by

$$Q_{\text{MAX}} = \frac{[\text{SNR}]_{\text{MAX}}^2}{t} = \sum_j \frac{k (C_{tj} - C_{oj})^2}{(C_{tj} + C_{oj})} = \sum_j Q_j. \quad \text{A(19)}$$

The sum of figure-of-merit values for all channels is greater than Q in eq. (2) for one SCA which optimally brackets the photopeak, or which includes the entire spectrum.

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A FILM TECHNIQUE FOR DETERMINING THE ABSORBED DOSE AT EXIT FROM THE PATIENT DURING RADIATION EXPOSURE*

By

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A film technique to image patient therapeutic radiation exposure has been used successfully for treatment verification.¹ This technique simply involves placing a graphic arts film, namely, Kodak Fine Grain Positive film, under the patient throughout a complete radiation treatment. The films are of such quality that various anatomic structures, as well as variations due to patient thickness in the treatment field, are clearly distinguishable. In consequence, the absorbed dose at exit may vary appreciably over the treatment field. An investigation was undertaken to develop a method whereby the absorbed exit dose could be measured easily from the treatment film by the radiotherapist, radiological physicist, or technician.

Advantages of this technique for the radiotherapist include:

- a) Evaluation of the absorbed dose in areas where healthy tissue may have been irradiated unnecessarily due to improper shielding or patient motion.
- b) Evaluation of a change in exit dose as a patient gains or loses weight during the therapy period.
- c) Observation of daily or weekly changes in exit dose resulting from possible changes in source output.
- d) Evaluation of wide variations in exit dose from patients with large fields such as the Maltese cross setup for Hodgkin's disease. With this information, it may be possible to alter shielding or to incorporate compensators that result in a more selectively controlled dose across the treatment field.
- e) Estimation of exit dose behind shielding blocks protecting vital structures such as lung, larynx, and spinal cord.
- f) Retention of the verification film and corresponding calibration as a part of the patient record file for subsequent evaluation, and for comparison of evaluations from different radiotherapy centers. In addition, if the same patient was treated at different centers, an estimate of the absorbed exit dose received from these treatments could be made.

PROBLEMS IN FILM CALIBRATION

Of necessary importance for obtaining the absorbed exit dose from the treatment verification film is a proper calibration curve whereby measured densities on the film can be related to the absorbed exit dose. The most essential features in the use of photographic emulsions for dosimetry are proper film calibration and standardized processing techniques. To eliminate

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processing problems, all films must be developed simultaneously under well-controlled conditions.

The dependence of the sensitivity of photographic emulsions on the quality of radiation is a particular problem in film dosimetry. Although film sensitivity to high-energy radiation is quite uniform (such as exposure to ^{60}Co), this is generally not the case when film is directly exposed to radiation of low energies.² When the ^{60}Co beam passes through a phantom or patient, the electron spectrum within the medium as well as the photon spectrum reaching the film may be altered to such an extent that film sensitivity may change.² These factors could make the film response dependent upon field size and patient thickness. Therefore, to obtain a proper film calibration curve and to establish the validity of using the treatment verification film for determining the absorbed exit dose, we investigated the effects of field size and depth.

EXPERIMENTS

Film calibration to evaluate absorbed dose is based on measurements of exposure with an ionization chamber and calculations of the absorbed dose at depth. The cobalt-60 machine was used to expose films placed at specified depths in tissue-equivalent Masonite blocks which had previously been used to determine the tissue-air ratios (TAR) on that machine. The absorbed dose is calculated as

$$D_{\text{Depth}} = \text{TAR} \times X \times f, \quad (1)$$

where D_{Depth} is the absorbed dose (rads) in phantom, TAR is the tissue-air ratio, X is the exposure in roentgens, and f is the roentgen-to-rad conversion factor.

A calibrated Victoreen chamber was used to measure the exposure in roentgens. A correction was made for the equilibrium thickness factor, $A_{\text{eq}}^{3,4}$

Figure 1 illustrates the experimental setup. For convenience and simplicity, a field size of 10 cm x 10 cm at a depth of 2.2 cm was selected as the standard. Masonite blocks of 7 cm thickness were added for backscatter. By means of eq. (1), absorbed doses ranging from 10 to 160 rads were selected to define 8 points on the calibration curve. A 35 mm x 12 in. (30.48 cm) film packet was inserted at the 2.2 cm depth, and the appropriate exposure was given to each of the 8 film packets. The films were developed in Kodak Rapid X-ray Developer for 5 minutes at 68° F (20° C), using nitrogen burst agitation. The same procedure was used to expose films at field sizes ranging from 30 cm² to 300 cm², at depths of 2 cm and 20 cm. A standard calibration was always run as a control on development. Film densities were measured on a Macbeth Quantalog densitometer. Figure 2 shows a characteristic curve of net density as a function of log absorbed dose for the various field sizes and depths of interest, compared to the standard curve. The total variation of the absorbed dose from the standard curve was within ± 5 per cent. These results indicate that the response of this film is independent of depth and field size. A single calibration curve can, therefore, be used to determine absorbed dose.

A similar experiment was performed with the Van de Graaff generator. This machine operates with a broad x-ray spectrum at a peak energy of 2 MeV. Depth-dose curves were used to calculate entrance, tumor, and exit dose for patient setups on the machine. The depth-dose curves were determined utilizing the same tissue-equivalent Masonite blocks. The absorbed dose at depth on the Van de Graaff machine is calculated as

$$D_{\text{Depth}} = X \times f \times \%DD, \quad (2)$$

Figure 1. Illustration of the experimental setup used for film calibration.

where D_{Depth} is absorbed dose (rads) in phantom, X is the exposure in roentgens, f , the roentgen-to-rad conversion factor, and %DD, the per cent depth dose.

Values for appropriate backscatter and displacement factor corrections were used.^{3,4} In addition, an inverse-square correction for maximum buildup level was necessary. Similar field size and depth parameters were investigated using a 2.2 cm depth and a 10 cm x 10 cm field as standard. The experimental setup was basically the same as in Figure 1, except that the source-to-depth distance was 100 cm rather than 81.6 cm. Processing conditions were the same as in the previous experiment. Agreement was again obtained, the total variation being ± 5 per cent of the standard. Thus the correct absorbed dose could be obtained from this film with the Van de Graaff generator.

An additional standard calibration experiment was performed with the patient treatment table instead of the 7 cm Masonite block as the backscatter medium. A negligible film exposure difference was noted. Therefore, in calibration exposures for the evaluation of treatment verification films, the table could be used as backscatter material since it serves this function during actual patient exposure.

From the experimental results obtained on the cobalt-60 and Van de Graaff machines, a standard film calibration curve can be obtained to determine the absorbed dose. The standard technique of 10 cm x 10 cm field and 2.2 cm depth can be used to determine the absorbed dose at exit from the patient verification film.

Figure 2. Characteristic curve showing independence of film response from field size and depth for ⁶⁰Co. Data for the 10 cm x 10 cm field and 2.2 cm depth are used as a standard and are drawn as a solid curve.

PATIENT FILMS

Cobalt-60 machine treatment verification films of a patient being treated in the pelvic area were exposed, the field size at tumor depth being 17 cm x 17 cm. The measured patient thickness ranged from 18 cm in the lower abdomen to 24 cm in the middle of the abdomen. The verification film was placed in a thin General Electric Flexible X-ray Film Holder (without lead backing). The flexible cassette was maneuvered so as to be in good contact with the patient's skin over the treatment area. Calibration films were exposed on the cobalt-60 machine by the standard technique. Figure 3 shows the calibration curve as a plot of net density vs absorbed exit dose in rads. On the pelvic treatment verification film in Figure 4, the letters A and B denote patient thickness areas of 18 cm and 24 cm, respectively. From depth-dose charts the exit doses were estimated to be 45 and 38 rads, respectively. The values correlating the measured film densities in areas A and B with the absorbed exit dose from the curve in Figure 3 were 47 and 36 rads, respectively. For comparison, several thermoluminescent dosimeters (TLD) were placed on the patient in area A. The average dose measurement from the TLDs was 48 rads.

Treatment verification films were also taken of a patient with Hodgkin's disease, utilizing a Maltese cross setup with shielding blocks in a large field. This patient was treated on the Van de Graaff machine (Figure 5). The absorbed exit dose, obtained from the verification film with a calibration curve, ranged from 41 to 80 rads. This range is due to the various anatomic structures and differences in patient thickness within the treatment field. The absorbed exit

Figure 3. ^{60}Co calibration curve for pelvic treatment verification film.

dose behind the lung shielding blocks was estimated to be from 5 to 15 rads.

Other treatment verification films of patients treated on the cobalt-60 and Van de Graaff machines were made and used with the standard calibration curve; the results were similar to those shown in Figures 4 and 5.

CONCLUSION

Experiments utilizing the ^{60}Co and the Van de Graaff generator to expose treatment verification film, namely, Kodak Fine Grain Positive, at various field sizes and depths have shown that it is easy to obtain a standard calibration curve which relates measured densities on the patient verification film to absorbed exit doses. The following four steps should be followed:

- a) For the film calibration, a tissue-equivalent material such as Masonite should be used for the buildup equilibrium layer. Approximately 10 film packets should be exposed to include absorbed doses ranging from 5 to 150 rads. These points will adequately define a characteristic curve.
- b) A flexible vinyl cassette should be used with the patient verification film, so that the cassette will be in contact with the patient's skin.
- c) Proper processing techniques are always necessary for accurate dosimetry work. Calibration films should always be processed together with the treatment verification film. Because of possible latent-image fading, the calibration film should be exposed on the same day as the treatment film, even though it was found that the error when the films were developed five days after exposure was negligible.
- d) A properly calibrated densitometer should be used to measure film densities.

Initial studies indicate that, with this technique, measurements of the patient exit dose can be obtained easily by the radiotherapist, radiological physicist, or technician. Possible uses for these measurements include determination of absorbed exit dose in areas where healthy tissue

Figure 4. Pelvic treatment verification film. A and B are areas where absorbed exit doses obtained from the calibration curve in Figure 3 are 47 and 36 rads, respectively. Estimated absorbed exit doses in these areas from depth-dose charts are 45 and 38 rads, respectively.

Figure 5. Treatment verification film for a patient with Hodgkin's disease. A, B, and C are areas where absorbed exit doses obtained from a calibration curve are 41, 50, and 80 rads, respectively. Estimated exit doses behind the lung shielding blocks ranged from 5 to 15 rads.

has been irradiated unnecessarily, observations of changes in exit dose as the patient gains or loses weight, detection of changes in source output, alteration of shielding blocks or incorporation of compensators for more selectively controlled dose across the treatment field, estimation of exit dose behind shielding blocks, and evaluation of similar treatment arrangements in other radiotherapy departments. This information, coupled with the visual information obtained from the recorded patient treatment verification film, can be of use to the radiotherapist in enhanced quality control.

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DOSE COMPUTATION METHODS FOR 4- AND 6-MeV X RAYS*

By

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An ad hoc subcommittee of the Special Committee on Radiation Dosimetry of the American Association of Physicists in Medicine has reviewed the several different methods described in the AAPM Quarterly Bulletin and in the open literature for calculating tumor dose when high-energy x-ray beams are employed. Because of the advantages inherent in the use of tissue-air ratios (TAR), specific attention was given to the extension of the TAR concept to x-ray energies higher than 2 MeV.

The TAR concept¹ has been widely used in cobalt-60 and 2-MeV x-ray dosimetry. This concept allows the separate computation of the dose due to the primary beam and the dose resulting from phantom or patient scatter. Hence it is possible to calculate the total dose to any point within the irradiated volume, or to points within the blocked volume, when irregular fields of various shapes and sizes are utilized.

It seems appropriate, therefore, to extend the use of the TAR concept to higher-energy x rays. Up to the present time, TAR have been measured for 4- and 6-MeV x rays. The ratio requires the measurement of dose to an "equilibrium mass" of tissue placed at the isocenter in air. This is adequately achieved by using a calibrated, small-volume ionization chamber within a miniphantom having the dimensions of the equilibrium mass. The chamber behaves as a Bragg-Gray cavity within this miniphantom, and the dose can therefore be calculated by the use of the appropriate C_λ [§] factor. The TAR, however, is obtained by merely taking the ratio of the ionization chamber readings measured with the chamber in the depth within the large phantom and with the chamber in the miniphantom. The recommended calibration procedure for a 4- or 6-MeV x-ray beam is the measurement of the absorbed dose at the depth of maximum buildup, i.e., at 5 cm depth in a large phantom. However, the dose at a point on the central ray, located at the isocenter, can be calculated from

$$D_{\text{isocenter}}(d, A_d) = \frac{D(d_{\text{cal}}, A_{\text{cal}})}{\text{TAR}(d_{\text{cal}}, A_{\text{cal}})} \times G \times \text{TAR}(d, A_d),$$

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[§] C_λ is the factor by which a dose in a medium can be determined from a measurement of ionization in air or a gas. The factor depends on the ratio of average stopping powers of the ion chamber wall to air for cobalt-60 gamma rays and the radiation of interest, effective energy absorption coefficients, a displacement factor, a calibration factor of the ion chamber when calibrated for cobalt-60 gamma rays, and the roentgen-to-rad conversion factor, f , for the tissue.

where D is the dose in rads, d is the thickness of tissue between the surface and the position at which the dose is to be calculated, A_d is the area of the field at the level of the dose calculation, d_{cal} is the calibration depth (d_{max} or 5 cm), A_{cal} is the area of the field at the calibration depth, and G is a collimator factor normalized to the calibration field size. It should be noted that G has to be obtained for each machine individually. This factor may be derived by dividing the doses at the depth of the dose maximum for various field sizes by the corresponding TAR, also at the depth of the dose maximum:

$$G = \frac{D(d_{max}, A)}{TAR(d_{max}, A)} \bigg/ \frac{D(d_{max}, A_{cal})}{TAR(d_{max}, A_{cal})}.$$

The tissue-maximum ratio (TMR) concept,² which is a special case of the tissue-phantom ratio (TPR) concept,³ is applicable to isocentric dosimetry only when fields of regular shape are employed. This restriction stems from the fact that, by definition, the TMR at the depth of the dose maximum is unity for all field sizes. This is in contrast to the TAR which has only one point of normalization; that point is at "zero" field size.

By definition, tissue-maximum ratios are related to tissue-air ratios by the backscatter factor (BSF):

$$TMR(d, A) \times BSF(d_{max}, A) = TAR(d, A).$$

Since the backscatter factor is the same as $TAR(d_{max}, A)$,

$$TMR(d, A) = \frac{TAR(d, A)}{TAR(d_{max}, A)}.$$

In general, as the energy of the x-ray beam increases, phantom backscatter decreases; thus the BSF gradually approaches unity and hence is less dependent on field size. When the BSF has the value 1.00, the quantities TMR and TAR become equal. This situation does not obtain, however, for 4- and 6-MeV x-ray beams. Measurements of BSF have been made and found to be comparable to, and in some instances somewhat greater than, the BSF for cobalt-60 gamma rays.⁴

Tissue-output ratios (TOR), by definition,⁵ contain variations of collimator scatter with field size, and hence the measured values are likely to differ from machine to machine. These differences may be small and of little clinical significance. However, in the interest of uniform and precise dosimetric practice, the tabulation of tissue-output ratios, measured on one machine, for general adoption should be discouraged.

In the interest of uniform and accurate dosimetric methods, the following recommendations are made for 4- and 6-MeV x-ray beams.

A. Isocentric Techniques

The use of either TAR or TMR in conjunction with isocentrically normalized isodose curves is recommended for the calculation of dose in moving-field and multiple-port isocentric treatments.

B. Irregular Fields

The use of TAR and scatter-air ratios (SAR) is recommended for the calculation of dose to any point within the irradiated volume. The SAR also allow the calculation of dose to points outside the irradiated volume.

C. Fixed SSD Techniques

Dose distributions obtained under conditions that simulate an actual patient treatment, e.g., field size measured at the depth of $d_{\max}$ and constant SSD, remain the basic tools for determining the dose distributions in patients so treated. These dose distributions are frequently normalized to 100 per cent at $d_{\max}$, so that backscatter and collimator factors must be applied to obtain the integrator setting that will deliver the prescribed dose.

To facilitate precise and uniform dosimetric practices with 4- and 6-MeV x-ray beams, tables of tissue-air ratios, scatter-air ratios, and tissue-maximum ratios may be obtained from the Radiological Physics Center (RPC)* of the American Association of Physicists in Medicine. These tables have been verified by the staff of the RPC and are routinely used in their dosimetry evaluation program for clinical radiotherapy trial studies. It is highly recommended that spot check measurements of these quantities be made on the particular machine used for treatment, to verify the validity of the use of the published tables.

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STAGING AND TREATMENT OF ENDOMETRIAL CARCINOMA*

By

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The best primary therapy for adenocarcinoma of the uterine corpus is still debatable. Published series of cases and survival data are often difficult to compare because of differences in staging criteria and bias in the selection of treatment. The adequacy of surgical and radiotherapeutic techniques further complicates proper evaluation. The purposes of this paper are to illustrate the problems of clinical staging as a means of assessing the extent of disease and to examine the role of radiotherapy in the treatment of endometrial adenocarcinoma.

MATERIALS AND METHODS

Two hundred eighty-seven patients with adenocarcinoma of the endometrium (including 13 with adenoacanthoma) received primary therapy at the Chicago Lying-in Hospital, University of Chicago, between January 1, 1950 and December 31, 1970. Data on these patients were obtained by review of their hospital charts.

The mean age of the patients was 57 years, ten being below the age of 40 (the youngest 19 years) and four being over 80 years. Two hundred thirty-six (82 per cent) were white and 52 (18 per cent) were black. Eighty-two were nulliparous (29 per cent) and 205 were parous (71 per cent). According to the recorded opinion of the attending physicians, 57 (20 per cent) were premenopausal and 230 (80 per cent) were postmenopausal.

Reports of vaginal cytologic studies were available in only 141 patients (40 per cent). Class I or II smears (Papanicolaou classification) were reported in 99 patients, Class III in 26, Class IV in 13, and Class V in 3. Thus, in 30 per cent of patients in whom studies were obtained, suspicious or positive smears were found.

STAGING

A clinical stage was assigned to each patient after review of the chart, according to the system approved (1961) by the International Federation of Gynaecology and Obstetrics and The American College of Obstetricians and Gynecologists (1962). This staging system is as follows:

Stage 0	Carcinoma in situ
Stage I	Carcinoma confined to the corpus
Stage II	Corpus plus cervix involvement

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Stage III	Extension outside the uterus, but not outside the pelvis
Stage IV	Carcinoma involves bladder or rectum or extends outside the pelvis

By clinical staging the majority of patients fell into Stage I. In this series exploratory laparotomy comprised part or all of the primary therapy in 242 cases (84 per cent). Using the additional information from operative findings and pathology reports, a new "extended clinical staging" (ECS) was determined, based on the same definitions (see above) as those used for clinical staging. Of the 242 patients who had ECS, 54 (22 per cent) were found to have a different stage than had been previously determined. Generally, the extended clinical stage was more advanced (Table 1).

Table 1

COMPARISON OF CLINICAL STAGING AND EXTENDED CLINICAL STAGING

	Clinical staging		Extended clinical staging	
	Number	%	Number	%
Stage 0	0	0	18	7.4
Stage I	233	96.3	181	74.8
Stage II	1	0.4	6	2.5
Stage III	5	2.1	23	9.5
Stage IV	3	1.2	14	5.8
TOTAL	242	100.0	242	100.0

TREATMENT

Treatment varied according to the individual preference of the patient's attending gynecologist. Unless the patient's medical condition precluded operation, total hysterectomy and bilateral salpingo-oophorectomy was usually the treatment of choice, with or without radiotherapy. The modalities of radiotherapy consisted of (1) preoperative introductory radium (Heyman packing technique) to 5000 mg-hours; (2) postoperative application of a radium plaque to the vaginal apex (Stockholm applicator) to 1400 mg-hours; (3) external radiotherapy by means of 250 keV or cobalt teletherapy, with a dose of 4000 rads (or the maximum tolerated dose if less than 4000 rads) to the pelvis. In some instances more than one of these techniques was used (Table 2).

FOLLOWUP

Post-treatment followup has been either by routine clinic visits or by contact from the Registry of Neoplastic Diseases of the University of Chicago Hospitals and Clinics. The Registry has maintained 100 per cent contact with either the patient or her present physician. Five-year survival statistics have been calculated only for the 233 patients treated between 1960 and 1965. Nonsurvivors have been considered to include all who died, whether from this cancer or from any other cause.

Table 2
TREATMENT - ALL STAGES

Type of therapy	Number	%
Preoperative intracavitary radium, operation	36	12.5
Preoperative intracavitary radium, operation, post-operative vaginal plaque	19	6.6
Preoperative intracavitary radium, operation, post-operative external therapy	6	2.1
Operation only	80	27.9
Operation, postoperative vaginal plaque	54	18.8
Operation, postoperative external therapy	43	15.0
Operation, postoperative vaginal plaque, postoperative external therapy	4	1.4
Radiotherapy only (various techniques)	36	12.5
No operation or radiotherapy	9	3.2
TOTAL	287	100.0

RESULTS

In patients considered to have Stage I disease by extended clinical staging (Table 3), the five-year survival rate following operation alone was not significantly different from that following operation plus radiotherapy. Patients receiving radiotherapy alone (chiefly those who were poor operative risks) had less chance of surviving, but even so 13 of 21 did well. Three patients who received neither operation nor radiotherapy survived for five years. Two had diagnostic dilatation and curettage only and refused treatment. In the third patient carcinoma was found in an endometrial polyp, and no further treatment was advised.

Table 3
RESULTS OF TREATMENT - STAGE I

Type of therapy	Number	5 Year survival		Recurrence	
		Number	%	Number	%
Operation only	45	41	91.0	3	6.7
Operation and radiotherapy	98	89	90.7	4	4.1
Radiotherapy only	21	13	62.0	8	38.1
No operation or radiotherapy	6	3	50.0	3	50.0
TOTAL	170	146	85.9	18	10.6

Proven recurrences were found in a slightly smaller percentage of patients receiving radiotherapy in addition to operation. However, the type of radiotherapy used did not seem to be important. Not enough patients were treated with intracavitary radium and/or postoperative radium plaques to permit any statistically significant conclusions regarding the type of radiotherapy most effective in reducing recurrences.

In Stage I patients treated by operation alone (45 patients) or by operation plus radiotherapy (98 patients), the depth of myometrial invasion by the tumor influenced survival (Table 4). The

Table 4
DEPTH OF TUMOR INVASION INTO MYOMETRIUM - STAGE I

	Number	%	5 Year survival		Treatment	
			Number	%	Operation	Operation and radiation
No invasion	89	62	87	98	28	59
Superficial invasion	31	22	28	90	9	19
Deep invasion	23	16	15	65	4	11
TOTAL	143	100	130	91	41	89

pathologists' criteria for reporting deep myometrial invasion generally followed the rule that penetration through one-half the thickness of the myometrium or more constituted deep invasion. From these data it was not clear whether operation plus radiotherapy was better than operation alone for patients whose tumor had invaded the myometrium.

Very few patients had Stage II disease (Table 5). The three patients who survived received operation plus external radiotherapy.

Stage III patients were generally treated by operation followed by external radiotherapy to the pelvis. Of the 11 patients surviving for five years, nine had external radiotherapy to the pel-

Table 5
RESULTS OF TREATMENT - STAGE II

Type of therapy	Number	5 Year survival		Recurrence	
		Number	%	Number	%
Operation only	1	0	0	1	100
Operation and radiotherapy	4	3	75	2	50
Radiotherapy only	2	0	0	2	100
No operation or radiotherapy	0	0	0	0	0
TOTAL	7	3	43	5	72

vis. One had intracavitary radium preoperatively and one had a postoperative radium plaque to the vaginal apex. One patient is now alive twelve years after operation alone with no added radiotherapy. In the three patients who were not operated, various combinations of radium and external radiotherapy were used. Again, the small number of cases treated in this stage makes it difficult to discern any trend in the results (Table 6).

Table 6
RESULTS OF TREATMENT - STAGE III

Type of therapy	Number	5 Year survival		Recurrence	
		Number	%	Number	%
Operation only	1	1	100	0	0
Operation and radiotherapy	19	11	58	9	48
Radiotherapy only	3	2	67	2	67
No operation or radiotherapy	1	0	0	1	100
TOTAL	24	14	58	12	50

Regardless of the type of therapy given to patients with Stage IV disease, the results were uniformly discouraging. The only patient who survived five years was found on abdominal exploration to have a positive paraaortic node. She received total hysterectomy and bilateral salpingo-oophorectomy with postoperative external radiotherapy to the pelvic nodes and to the paraaortic nodes up to the diaphragm. She is now alive and well 12 years later (Table 7).

Table 7
RESULTS OF TREATMENT - STAGE IV

Type of therapy	Number	5 Year survival		Recurrence	
		Number	%	Number	%
Operation only	4	0	0	4	100
Operation and radiotherapy	7	1	14	6	86
Radiotherapy only	4	0	0	4	100
No operation or radiotherapy	2	0	0	2	100
TOTAL	17	1	6	16	94

Of the patients who were found to have Stage 0 disease (carcinoma in situ) as a result of extended clinical staging, all did well. Operation was used as the primary treatment, and the addition of radiotherapy appeared to be superfluous (Table 8).

Table 8
RESULTS OF TREATMENT - STAGE 0

Type of therapy	Number	5 Year survival		Recurrence	
		Number	%	Number	%
Operation only	13	13	100	0	0
Operation and radiotherapy	2	2	100	0	0
Radiotherapy only	0	0	0	0	0
No operation or radiotherapy	0	0	0	0	0
TOTAL	15	15	100	0	0

DISCUSSION

The findings reported show that a large proportion of patients with adenocarcinoma of the uterine corpus seen at Chicago Lying-in Hospital had early disease. Moreover, with total hysterectomy and bilateral salpingo-oophorectomy used as the basis for treatment, over 90 per cent of these patients did well. However, four important questions arise. First, does the technique of extended clinical staging used here throw any light on the whole question of staging of disease? Second, in Stage I disease, does the slightly lower (though not significant) incidence of recurrence in patients who received additional radiotherapy (4.1 per cent) compared with those who received operation alone (6.7 per cent) justify the use of such radiotherapy routinely? Third, is the presence of deep myometrial tumor invasion an indication for postoperative radiotherapy? Finally, can any conclusions be drawn with regard to the best treatment for patients with advanced disease?

Dissatisfaction has frequently been expressed about the international system of staging in carcinoma of the corpus. One of the chief reasons has been the large proportion of cases found to fall into Stage I. This is still true even when extended clinical staging is used. Gusberg¹ has indicated the importance of the size of the uterus and the degree of differentiation of the tumor, and has proposed a new staging system on this basis. These additional criteria have led us to divide Stage I, on a tentative basis, into:

- Stage Ia Normal-sized uterus, well differentiated tumor
- Stage Ib Normal-sized uterus, poorly differentiated tumor
- Stage Ic Enlarged uterus, well differentiated tumor
- Stage Id Enlarged uterus, poorly differentiated tumor

This breakdown of Stage I has been used only since 1966, and insufficient data have been accumulated from which to draw any conclusions. It was not possible to extract enough information from the older records to extend this staging modification retrospectively. The use of extended clinical staging points up the deficiencies of the usual staging, but does not help in breaking down Stage I into useful divisions.

The value of neither preoperative intracavitary radium nor of a postoperative vaginal radium plaque can be demonstrated in this series. The basic importance of operative treatment suggests that the varying delay and the previously reported theoretical benefits of postoperative

intracavitary radium may be illusory.²⁻⁷ Likewise, the reports of benefit from preoperative external radiotherapy to the pelvis as advocated by some authors are perhaps unfounded.⁸ The merits of postoperative vaginal radium plaques, however, cannot be dismissed so easily. We do not suggest that surgery alone is adequate therapy for all patients, as has been proposed by others.⁹ Recurrence in the vagina has been reported in as many as 15 per cent of patients treated solely by operation.^{10,11} For this reason, it has been our practice in recent years to use a postoperative vaginal radium plaque routinely. Further refinement of the staging for early lesions may help in the evaluation of this technique.

The presence of deep myometrial invasion by tumor increases the probability of lymphatic tumor spread. Published series show the incidence of positive lymph nodes in patients undergoing radical hysterectomy with either selective or total lymphadenectomy to range from 12 per cent to 14 per cent in Stage I disease.^{12,13} Although the data in Table 4 are not conclusive, the poorer results when the myometrium is deeply invaded suggest that postoperative external radiotherapy may be useful in these cases to treat the possibly involved pelvic nodes. The small fraction of patients with deep myometrial invasion (16 per cent) does not warrant the indiscriminate administration of external cobalt teletherapy to all patients, but suggests its advisability in those found at operation to have deeply invasive disease.

Management of the 31 patients with Stage II and Stage III disease varied. No conclusions can be drawn about the efficacy of any one method, except that some combination of operation and radiotherapy is probably appropriate. There were surprisingly few patients in Stage II. This perhaps emphasizes the importance of thorough initial evaluation, including endocervical curettage, if Stage II lesions are to be considered as a separate entity. When a patient has clinically apparent Stage II or Stage III disease prior to operation, our present management is to use external cobalt teletherapy preoperatively to the pelvis, followed by total hysterectomy and bilateral salpingo-oophorectomy. Following operation a radium plaque is applied to the vaginal apex. In the instances where operation is performed for a supposed Stage I lesion and more extensive disease is found, making the case Stage II or Stage III by extended clinical staging, external radiotherapy is given postoperatively.

The prognosis for any patient with a Stage IV lesion is poor. Where the disease is widespread, local radiotherapy may be used to relieve discomfort or to stop bleeding if this is a problem. In view of recent reports on the beneficial effects of progesterone in some cases, this therapy should be used for all patients with Stage IV disease.¹⁴⁻¹⁶ The present series contains 20 patients in whom this treatment was used, four of whom were noted in their charts to have had a "response" to this treatment.

CONCLUSIONS

1. Two hundred eighty-seven patients with adenocarcinoma of the corpus uteri were treated at the Chicago Lying-in Hospital between 1950 and 1970. Five-year survival data are available for 233 patients.

2. By traditional clinical staging, 96 per cent of the patients fell into the generally accepted international Stage I. The use of a new system of extended clinical staging decreases this number to 75 per cent. It is apparent that the staging evaluation of these patients needs further refinement.

3. Operative treatment by total hysterectomy and bilateral salpingo-oophorectomy appears

to be the best primary therapy currently available for most patients with Stage I disease. There is suggestive evidence for the value of adding a postoperative vaginal radium plaque, and in those cases with deeply invasive tumors there may be value in adding postoperative cobalt teletherapy to the pelvis.

4. For patients with Stage II or Stage III disease, various combinations of radiotherapy and operation are advisable, while for those with Stage IV disease local radiotherapy as indicated and systemic progesterone therapy are appropriate.

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BIOCHEMICAL CORRELATES OF CARDIAC HYPERTROPHY V. LABELING OF
COLLAGEN, MYOSIN AND NUCLEAR DNA DURING EXPERIMENTAL
MYOCARDIAL HYPERTROPHY IN THE RAT*

By

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The rapid enlargement of adult rat heart that follows experimentally produced pressure overload results from hypertrophy of muscle fibers and hyperplasia of connective tissue cells. The increased activity of connective tissue cells in cardiac hypertrophy is well documented both by radioautography and by determination of mitotic indices. Cardiac enlargement produced by systemic hypertension¹ or by increased resistance to blood flow produced by construction of the ascending aorta²⁻⁴ results in marked labeling, with [³H]thymidine, of otherwise inactive connective tissue nuclei. The mitotic indices of interstitial cells of hypertrophied hearts were found to be ten times higher than in controls.⁵ Both the mitotic activity and the degree of DNA labeling are proportional to the degree of hypertrophy.^{4,5}

Nuclear DNA synthesis in enlarging adult hearts is almost exclusively confined to the interstitial tissue. The very rare labeling of muscle cell nuclei with [³H]thymidine seen in radioautographs of the hypertrophic heart^{4,6} may be related to the increased polyploidization of existing nuclei. The frequency of polyploid nuclei is considerably increased in pathological specimens of hypertrophied human hearts,^{7,8} but only a relatively small increase in polyploidy is seen in experimentally produced cardiac hypertrophy.^{5,9}

A substantial increase in collagen content has been noted in hearts enlarging in response to constriction of the ascending aorta⁴ or of the main pulmonary artery.¹⁰ Collagen is believed to be produced only by connective tissue cells. It is apparent, therefore, that synthetic activities of both muscle and connective tissue cells are stimulated in adaptive cardiac growth.

It is our purpose to present information concerning the significance of connective tissue hyperplasia and increased collagen synthesis in the development of cardiac hypertrophy. We examined the time courses of enhancement of some of the synthetic activities of connective tissue cells and muscle cells following experimental aortic constriction in the rat. Connective tissue hyperplasia was measured by incorporation of [³H]thymidine into purified nuclear DNA. Collagen biosynthesis in connective tissue cells was estimated by following the incorporation of radioactive proline into tissue hydroxyproline, since collagen is the only protein in mammalian tissue contain-

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ing significant amounts of hydroxyproline. Collagen hydroxyproline is derived from proline that is hydroxylated after its incorporation into protocollagen, the polypeptide precursor of collagen.¹¹ Muscle cell synthetic activity was monitored by measuring the incorporation of labeled amino acids into myosin.

Our results show that the labeling of collagen is increased several times by the second post-operative day. Its maximal labeling exceeds that of the bulk of noncollagenous protein and myosin, and precedes that of nuclear DNA by several days. After this early burst of activity, a moderately increased rate of labeling was noted as long as one month after aortic constriction.¹²

MATERIALS AND METHODS

Production of experimental cardiac hypertrophy. The ascending aortas of female Sprague-Dawley rats weighing 195-205 g were constricted with silver bands, as previously described.¹³ Rats with sham operations served as controls. The animals were killed at varying times after operation. Hearts were removed, rinsed in ice-cold 0.01M Tris buffer, pH 7.5, containing 0.25M sucrose, 0.01M KCl, 0.1M Na-EDTA, and 10 USP units of heparin/ml, and the atria and aortas were trimmed off. The hearts were then blotted, weighed, and analyzed as described below.

Administration of radioisotopes. Radioisotopes were administered intraperitoneally at varying times after aortic constriction. In experiments comparing the synthesis of collagen and noncollagenous protein, each animal received 50 μ Ci of [2,3-³H]proline unless otherwise noted. For the simultaneous measurement of labeling of collagen, myosin, and DNA, 50 μ Ci of [2,3-³H]proline, 10 μ Ci of [U-¹⁴C]lysine, and 100 μ Ci of [methyl-³H]thymidine were administered together. Preliminary experiments showed that, when [³H]proline and [³H]thymidine were injected individually, the DNA fraction contained no radioactivity derived from [³H]proline, and that the fraction used to assay for hydroxyproline contained no detectable radioactivity derived from [³H]thymidine. Similarly, label derived from [¹⁴C]lysine was not present in significant amounts in the hydroxyproline or DNA fractions. The labeling of myosin and noncollagenous protein was also compared using [³H]proline. Animals were killed 3 hours after injection of the radioisotopes, since incorporation into the components was comparable 3, 6, and 12 hours after injection.

Determination of amount and radioactivity of collagen. The hearts were frozen in liquid nitrogen, pulverized, and extracted twice in hot 5 per cent trichloroacetic acid (TCA) by a procedure in which over 95 per cent of the tissue collagen is recovered.¹⁴ An equal volume of concentrated HCl was added to an aliquot of TCA extract, and the solution was hydrolyzed in an autoclave overnight. The content and radioactivity of hydroxyproline in the hydrolysate were determined by methods previously described,^{15,16} with slight modifications. Hydroxyproline in the hydrolysate was oxidized to pyrrole. The pyrrole was extracted into toluene, and samples were taken for pyrrole determinations and for assay of radioactivity in a Packard Tri-Carb liquid scintillation counter. [³H]Toluene standards were counted simultaneously to convert counts per minute to disintegrations per minute. Under the conditions of these experiments, pyrrole recovery was proportional to the amount of hydroxyproline in the hydrolysate of TCA extract. The collagen content of the heart was calculated from a standard curve prepared from hydrolysates of calf skin gelatin. Since cardiac DNA and protein concentrations do not change significantly during the first two weeks after aortic constriction,⁵ radioactivity determinations are expressed as dpm per 100 mg tissue. Collagen content was also measured by the method of Neuman and Logan,¹⁷ as previously described.⁴

Determination of noncollagenous protein radioactivity. The residue remaining after extraction with hot 5 per cent TCA was left overnight at 4° C in 0.1N NaOH. After centrifugation at 3000 x g, the supernatant fluid was collected and neutralized with 1N HCl, and 0.1 volume of 50 per cent TCA was added. After 30 min. in ice, the precipitate was collected and washed 3 times with 5 per cent TCA at room temperature. The residue was extracted for 30 min. at 80-90° C with 5 per cent TCA, washed with acetone, and then with absolute ethanol. The residue was dried under an infrared lamp and dissolved in formic acid. A sample was evaporated for protein determination.¹⁸ Another sample, containing approximately 1 mg of protein, was mixed with BBOT scintillation mixture (containing 5 g BBOT in 850 ml toluene and 150 ml methyl cellosolve) and counted in a Packard Tri-Carb liquid scintillation counter. An external standard was used to convert cpm to dpm.

Determination of myosin radioactivity. When the labeling of noncollagenous protein, collagen, myosin, and nuclear DNA was measured simultaneously, the following procedure was adopted: Hearts were washed, blotted, and weighed as above. Two to three hearts were then pooled, finely minced with scissors, and divided into two parts. Approximately 0.3 g was used for extraction of collagen and noncollagenous protein as described above, and the rest, for the preparation of myofibrils and nuclei by a modification of the procedure described previously.¹⁹ Minced heart tissue was homogenized 1-1/2 min. in 0.3M sucrose, 0.01M KCl, 1 mM MgCl₂ in an MSE homogenizer at top speed (8000 rpm) and centrifuged for 10 min. at 1000 x g. The pellet was suspended in 2.2M sucrose (final molarity) containing 1 mM MgCl₂, and centrifuged in the Spinco SW39 rotor for 30 min. at 140,000 x g. The content of the upper two-thirds of each tube was used for the preparation of myofibrils; the pellet contained crude nuclei. The supernatant was diluted to 0.3M sucrose and centrifuged for 10 min. at 1000 x g. The myofibrillar pellet was suspended in 0.1M KCl, 0.05M Tris-HCl buffer, pH 7.5, 0.5 mM Na-EDTA, 0.25 mM Na-thioglycolate, and again homogenized for 1-1/2 min. and centrifuged as described above. This step was repeated. The final pellet was suspended in 0.01M KCl, 0.05M Tris-HCl buffer, pH 7.5, 0.25 mM Na-thioglycolate, 0.01 per cent Triton X-100, using 10 strokes in a Dounce homogenizer (piston A), and centrifuged 10 min. at 1500 x g. This step was repeated with 0.01 per cent Na-deoxycholate replacing Triton in the suspending solution. The myofibrils were washed twice in the solution containing 0.01M KCl, 0.05M Tris-HCl, pH 7.5, and 0.25 mM Na-thioglycolate.

Myosin was extracted as follows: The myofibrils were suspended in a solution which contained, after the addition of KCl, 8 mM K-phosphate buffer, pH 7.8, 1 mM Na-EDTA, 5 mM Mg-ATP, and 0.25 mM Na-thioglycolate; 2M KCl was then added gradually to a final concentration of 0.6M. The suspension was homogenized by 10 strokes in a Dounce homogenizer, left in ice for 20 min. and centrifuged 2 hours at 160,000 x g. The top two-thirds of the supernatant were removed and dialyzed against 0.01M KCl, 0.25 mM Na-thioglycolate. The precipitated myosin was collected by centrifugation and dissolved in 0.2M KCl, 0.01M Tris-HCl buffer, pH 7.8. Approximately 20 mg of myosin were adsorbed on a 15 x 2 cm DEAE-cellulose column and eluted by a linear KCl gradient.* The fractions containing myosin were combined, and the 35-45 per cent (NH₄)₂SO₄ fraction was collected. The purified myosin was washed 3 times with 5 per cent TCA at room temperature and counted by the procedure described above for noncollagenous protein.

The purity of myosin was evaluated by SDS-polyacrylamide electrophoresis, and by analysis

* The elution pattern was similar to those described by McCarl and Margossian.²⁰

of the sensitivity of ATPase activity to Mg^{2+} and Ca^{2+} . In addition, myosin was purified after adding to the homogenate possible contaminating proteins (sarcolemmal proteins, actin, and tropomyosin-troponin complex) which had been labeled with [carboxyl- ^{14}C]p-chloromercuribenzoate.²¹

Determination of nuclear DNA radioactivity. The crude nuclear fraction was suspended in 0.15M NaCl, 0.01M EDTA, 0.05M Na-phosphate buffer, pH 6.5, and extracted with an equal volume of phenol. DNA was precipitated with ethanol, washed with 75 per cent ethanol, dissolved in standard sodium citrate solution (0.15M NaCl, 0.015M Na citrate), and digested with RNase and pronase. The nuclear DNA was separated from remnants of mitochondrial DNA, after denaturation and renaturation by CsCl equilibrium centrifugation, and precipitated by TCA. Its radioactivity was measured in the liquid scintillation counter and its deoxyribose content was determined colorimetrically by the diphenylamine reaction.²²

Estimation of extracellular space. The extracellular space was assumed to be equal to the volume of distribution of sulfate.^{23,24} For determination of the extracellular space in vivo, $Na_2^{35}SO_4$ (5 μ Ci/100 g body weight) was injected intravenously immediately after the animals had been nephrectomized under ether anesthesia. After an equilibration period of two hours,²⁵ the animals were killed and the hearts removed, blotted, weighed, placed in 3 ml of 0.5N NaOH, and heated overnight in a water bath at 45°C to dissolve the tissue. Protein was precipitated from a 1 ml sample by adding an equal volume of 10 per cent TCA.

Blood was collected in beakers containing heparin at the time when the animals were killed. The plasma was separated by centrifugation and diluted fivefold with water. Protein was precipitated by adding an equal volume of 10 per cent TCA. Samples (0.2-0.5 ml) of plasma and heart TCA supernatants were added to 15 ml of Bray's scintillator and counted in a Packard Tri-Carb liquid scintillation counter. An internal standard was used to correct for quenching.

The sulfate space was calculated according to the following formula: sulfate space (μ l/g wet weight of heart) = $\frac{\text{counts/min. g}^{-1} \text{ wet weight of heart}}{\text{counts/min. } \mu\text{l}^{-1} \text{ of plasma}}$. The ^{35}S counts in plasma were corrected for a Donnan factor of 0.95.²⁶

Total tissue water. A small portion (50-100 mg) of heart muscle was placed in a previously weighed crucible, weighed, and dried to constant weight in an oven at 100°C. The tissue water was calculated from the loss in weight of the tissue, and expressed as μ l/g wet weight.

Determination of intracellular proline concentration and specific radioactivity. [3H]Proline was injected intravenously (25 μ Ci/100 g body weight), and the rats were killed after 3 hours. The hearts were removed, frozen, pulverized, and extracted 3 times with 1 ml of 5 per cent TCA, as described above for the study of collagen. Plasma was collected and prepared for liquid scintillation counting and counted as described for the study of extracellular space. Total proline was determined in the heart TCA extracts and in the plasma TCA supernatant fluids by the method of Troll and Lindsley.²⁷

The intracellular concentration of proline was calculated by the formula

$$C_I = \frac{C_T - F_E \times C_E}{F_I},$$

where C_I = concentration in intracellular water in μ moles/100 ml, C_T = concentration in extracellular fluid in μ moles/100 ml, F_E = fraction of the tissue water that is extracellular, and F_I = fraction of the tissue water that is intracellular.

RESULTS

Time course of labeling of collagen and noncollagenous protein. The relative rates of synthesis of collagen and noncollagenous protein during the development of cardiac hypertrophy were estimated by comparing the incorporation of [^3H]proline into collagen hydroxyproline with its incorporation into noncollagenous protein. Two sets of experiments were carried out. In the first, surgical aortic constriction was performed at various times (2, 4, 7, and 30 days) before isotope administration and death (Figure 1). In the second, surgery was performed on the same day and the animals were killed at intervals of 1 to 7 days later (Figure 3). The results of the first experiment are shown in Figure 1. Maximal incorporation of radioactive proline into collagen (565 per

Figure 1. Effect of banding on recovery of radioactive proline as collagen hydroxyproline and in noncollagenous protein. Each animal was injected with 10 μCi [$\text{U-}^{14}\text{C}$]proline on day 7 after banding, and with 50 μCi [$2,3\text{-}^3\text{H}$]proline on all other days. The height of the bars represents mean radioactivity recovered in hearts from rats with sham operations (white bars) and from banded rats (black bars); vertical lines represent 2 SE of the means. The number in each group is given at the base of the bars. Each difference between corresponding control and experimental groups is significant by Student's t-test with a P value of 0.01 or less. Here and in other figures, HYPRO = hydroxyproline, NCP = noncollagenous protein, PRO = proline.

cent above control values) occurred on the second day, the earliest time studied. Incorporation was still considerably elevated (245 per cent above control values) on the 30th day after surgery. The labeling of noncollagenous protein with [^3H]proline was maximal on the second day and remained elevated on the fourth and seventh days (Figure 1). Maximal noncollagenous protein la-

being was only 160 per cent greater than control values, compared with a 565 per cent elevation observed in collagen labeling.

The increment in labeling of collagen hydroxyproline varied directly with the extent of cardiac hypertrophy indicated by the increase in heart weight (Figure 2). There was considerable variation in the extent of cardiac hypertrophy within each group. Differences in animal weight

Figure 2. Correlation of heart weight with collagen labeling. Individual data from the experiments in Figure 1 are plotted. Open circles = sham operations; solid circles = banded; solid lines are the best fit to the equation for a straight line.

as well as other unknown and uncontrolled variables may contribute to this variation. To minimize these factors, we banded a large group of closely weight-matched animals on a single day. Incorporation of [^3H]proline into collagen hydroxyproline and noncollagenous protein was then measured 1 to 7 days after banding. As seen in Figure 3, collagen labeling averaged about twice the control values on day 1, reached a peak on day 4 (330 per cent greater than controls), and then declined on day 7. Incorporation into noncollagenous protein reached a plateau 65 per cent greater than control on the second day (Figure 3).

Extracellular space and intracellular proline pool. Since changes in the level of amino-acid incorporation into proteins can be affected greatly by changes in the size and specific activity of the precursor pool, estimations of free-proline specific activity and concentration in the heart muscle were made 2 and 5 days after aortic constriction. It should be pointed out that this measurement represents values for both cardiac muscle cells and connective tissue cells.

The total tissue water of the rat heart averaged $777 \pm 1.4 \mu\text{l/g}$ in both controls and banded animals. Cardiac hypertrophy resulted in a significant increase in extracellular fluid (24 per cent at 2 days, 17 per cent at 5 days after banding) as measured by $^{35}\text{SO}_4^{2-}$. The possibility that this increase may have been the result of increased permeability of the myocardium to

Figure 3. Time course of collagen and NCP labeling after banding. The mean radioactivity recovered in rats with sham operations (open circles, N=1-3) and banded animals (solid circles, N=3-5) is plotted. Vertical lines represent 2 SE of the means. Where comparisons between banded and control animals are possible, the differences are significant by Student's t-test with a P value of 0.02 or less.

SO_4^{2-} ions under conditions of cardiac hypertrophy has not been eliminated. Accordingly, all calculations of proline intracellular concentrations in banded animals were made using extracellular sulfate space values obtained in the controls. Calculations of intracellular proline pools in banded rats using sulfate space values obtained with banded animals give values for total intracellular proline concentration 7 per cent greater than those presented in Table 1. Calculations of specific activity of intracellular proline on the same basis resulted in values 3 per cent lower than those presented in Table 1.

There was no increase in intracellular proline 2 days after banding, but by day 5 a 38 per cent increase in total intracellular proline had occurred. The specific activity of intracellular proline was 40 per cent greater than in controls on day 2 after banding, and was slightly less than control in hearts 5 days after banding (Table 1). It is evident from these data that the changes in proline incorporation into myocardial collagen resulting from aortic constriction are much larger than the changes in the specific radioactivity of intracellular free proline.

Time course of labeling of collagen, myosin, nuclear DNA, and noncollagenous protein. Differences in the temporal responses in the stimulation of synthetic activities of muscle cells and of connective tissue cells during cardiac hypertrophy were assessed further. Incorporation of labeled precursors into collagen, myosin, nuclear DNA, and noncollagenous protein were measured simultaneously. At varying times after aortic constriction, sets of animals received [2,3- ^3H]proline to measure collagen labeling, [U- ^{14}C]lysine for myosin and noncollagenous protein labeling, and [methyl- ^3H]thymidine to measure nuclear DNA labeling. The animals were

Table 1

INTRACELLULAR FREE PROLINE CONCENTRATION AND SPECIFIC RADIOACTIVITY IN
HYPERTROPHIED RAT MYOCARDIUM

Operation	Extracellular space			Intracellular proline pool			
	No. of rats	Hypertrophy (%)	^{35}S space ($\mu\text{l/g}$ wet wt.)	No. of rats	Hypertrophy (%)	Concentration ($\mu\text{moles/100 ml}$ cell H_2O)	Specific radioactivity ($\text{dpm} \times 10^{-3}/\mu\text{mole}$ of proline)
Sham, 2 days	8		247 ± 10	5		93.64 ± 4.05	258 ± 29
Banded, 2 days	8	29 ± 5	307 ± 19 ($P < 0.02$)	4	20 ± 4	95.93 ± 5.86 (NS)*	365 ± 19 ($P < 0.02$)
Sham, 5 days	10		270 ± 9	4		89.50 ± 5.49	395 ± 24
Banded, 5 days	8	41 ± 7	315 ± 13 ($P < 0.01$)	5	26 ± 8	124.14 ± 8.31 ($P < 0.02$)	327 ± 27 (NS)*

Intracellular proline was calculated on the basis of extracellular space values obtained in controls (sham operations). Percent hypertrophy was calculated by comparison of ratios of heart weight to body weight of banded and control rats.

* $P > 0.05$.

killed 3 hours later. The hearts were divided into groups (2 or 3 hearts per group), depending whether they exhibited moderate hypertrophy (10-25 per cent weight gain) or severe hypertrophy (more than 25 per cent weight gain). Hearts showing less than a 10 per cent increase in weight were discarded. Hearts of rats with sham operations served as controls (4 hearts per sample). The time course of collagen labeling depended on the degree of hypertrophy (Figure 4).

Figure 4. Time course of synthesis of collagen, myosin, NCP, and DNA following aortic banding. Animals were injected with a mixture of [2,3- ^3H]proline, [U- ^{14}C]lysine, and [methyl- ^3H]thymidine 2, 4, and 7 days after banding. Hearts were weighed and pooled according to severe hypertrophy (solid triangles), moderate hypertrophy (solid squares), or no hypertrophy (sham operations, open circles). The result from each pool of 2-4 hearts is plotted.

In hearts with severe hypertrophy, collagen labeling was maximal at 2 days and remained elevated to the same extent at 4 or 7 days after banding. In hearts with moderate hypertrophy, collagen labeling peaked sharply (over 10 times the control value) on the fourth day. In light of the observation that the enhancement of collagen synthesis is correlated with the degree of hypertrophy (Figure 2), peak collagen synthesis in severely hypertrophied hearts may have occurred before our first measurement on the second day after banding. Incorporation of [^3H]thymidine into DNA peaked at 7 days (Figure 4), when it was 600 per cent greater in banded rats than in controls. On the 11th day, the DNA specific radioactivity was less than the maximal observed value. DNA labeling in moderately hypertrophied hearts paralleled that in severely hypertrophied hearts, although at a lower level.

The time courses of labeling of myosin and of noncollagenous protein with [^{14}C]lysine were similar. The extent of labeling was proportional to the increase in heart weight, and a plateau of incorporation was reached at about 4 days after banding (Figure 4). Identical patterns of labeling of noncollagenous proteins and of myosin were obtained by using either lysine or proline as

precursors. The elevated incorporation observed in hypertrophied hearts was of the same magnitude in each case. The ratios of specific radioactivity of noncollagenous proteins to myosin were identical in control and banded animals for both precursors (Table 2). Thus, the same response of the two protein fractions to banding was observed with both proline and lysine.

Table 2

COMPARISON OF THE LABELING PATTERN OF NONCOLLAGENOUS PROTEINS (NCP)
AND MYOSIN WITH PROLINE AND LYSINE

Operation	Hypertrophy (%)	Specific radioactivity of NCP Specific radioactivity of myosin	
		Proline	Lysine
Sham, 2 days	24 and 27	1.53 ± 0.12	1.05 ± 0.08
Banded, 2 days		1.56 ± 0.10	1.06 ± 0.07
Sham, 4 days	32		1.02 ± 0.06
Banded, 4 days			1.12 ± 0.11
Sham, 5 days	36	1.56 ± 0.08	
Banded, 5 days		1.50 ± 0.02	

Two separate experiments were performed, one using [^3H]proline (N=3) as precursor and a second with [^{14}C]lysine (N=6). Data are expressed as means ± SE. Per cent hypertrophy was calculated as for Table 1.

Changes in heart collagen concentration and content. In our previous report, the increase in heart weight resulting from aortic constriction occurred primarily during the first 2-3 days.¹³ In the present experiments, heart weight increased relatively little on the first day after banding, sharply from day 2 to day 4, and then gradually to day 7, the latest time point measured (Figures 3 and 4).

Total collagen did not increase substantially until the seventh day after banding, when it was elevated by 81 per cent (Figure 5). Mean total tissue collagen on day 11 was only 104 per cent higher than in the control. Collagen concentration (collagen weight/wet weight tissue) fell during the early period of cardiac hypertrophy, when the heart weight increased and total tissue collagen remained relatively constant. After the fifth day following aortic banding, collagen concentration was elevated compared to the controls.

DISCUSSION

The labeling of heart components was measured at varying times after the induction of cardiac hypertrophy by aortic banding. Appropriate radioactive precursors were injected intraperitoneally. Three hours later the animals were killed, and the radioactivity in tissue fractions was determined. There was a sharp peak of collagen labeling, 400-600 per cent greater than in controls, between 2 and 4 days after surgery. The extent and timing of the peak depended on the degree of hypertrophy. An increase in the capacity for collagen labeling persisted until 30 days after surgery, the latest time studied. The peak coincided with the onset of collagen accumulation in the enlarging heart. The bulk of the increase in heart collagen content occurred between days 4 and 7 of hypertrophy; thereafter, the rate of increase diminished.

Figure 5. Effect of banding upon total heart collagen and collagen concentration. Open circles represent means from rats with sham operations; solid circles, means from banded animals.

The shapes of the curves of collagen and nuclear DNA labeling as a function of time after onset of hypertrophy are similar. The peak of the increase in collagen synthesis, however, is greater than that of DNA, and precedes it by about 3 days. The curves of myosin and noncollagenous protein (which is about 30 per cent myosin) labeling are unlike those of collagen and DNA, rising moderately within the first 2 to 4 days to a plateau approximately 100 per cent greater than in controls.

A possibility that must be considered is that the differences observed in labeling patterns of myosin, collagen, and nuclear DNA represent changes in specific activities in the precursor pool (radioactive proline, lysine or thymidine), rather than changes in their rates of synthesis. With regard to DNA, we have shown previously that the rate of entry of free thymidine into heart and its subsequent turnover were not changed after aortic constriction.⁴ Moreover, the time course of labeling of nuclear DNA coincides closely with measurements of mitotic activity,⁵ and with the number of labeled nuclei measured by radioautography.²⁻⁴

The problem is more complex, however, with respect to amino-acid incorporation into proteins. A moderate increase (40 per cent) in [³H]proline specific radioactivity 2 days after aortic constriction returned to normal values at the fifth postoperative day. This change is small in comparison to the three- to tenfold increase in collagen labeling observed on the first 2-4 postoperative days. Moreover, Kimata et al²⁸ reported no change in [³H]lysine specific activity after aortic constriction in the rabbit. There are, however, other uncertainties involved in the interpretation of incorporation data even when specific activities of intracellular free amino acids are measured. The immediate protein precursor is not the free amino acid but aminoacyl transfer RNA, and several studies have suggested that the total specific radioactivity of intra-

cellular free amino acid may not necessarily represent the immediate precursor.^{29,30} Another complication in the interpretation of our data is that myosin and collagen are being synthesized in different cell types, i.e., muscle and connective tissue cells, leaving open the possibility of nonparallel changes in the precursor pool in the two cell types. Despite these limitations, considering the magnitude of the changes observed, it is likely that the differences in labeling patterns of collagen and myosin represent differences in patterns of synthesis.

The early increase in free proline specific activity and the later increase in the total proline pool is of interest because of previous observations that the rate of transport of amino-isobutyric acid is increased in hypertrophic heart³¹ and also in passively stretched rabbit papillary muscle.³²

A number of possible mechanisms that might explain the observed increase in collagen labeling following aortic banding require consideration. The increased tension resulting from volume overload could be followed by an increased synthesis of collagen. A number of tissues, such as the uterus stretched by wax, bone subjected to the pull of a muscle, and wounds healing under tension, respond to tension by an increased synthesis of collagen. Schreiber et al.,³⁴ however, have presented evidence that is somewhat in disagreement with our findings and opposed to the above interpretation. They found no increase in incorporation of [¹⁴C]proline into collagen hydroxyproline of hearts that were perfused under increased pressure. Incorporation of [¹⁴C]lysine into myosin was enhanced, however, by an increase in perfusion pressure.

Another possibility is that collagen or other hydroxyproline-containing polypeptides are specifically involved in regulation of muscle growth, as suggested by in vitro studies of myogenesis.³⁵ It is also possible that aortic constriction leads to relative cardiac ischemia that may serve as a stimulus for collagen synthesis. Proliferation of collateral arterioles, accompanied by increased DNA synthesis, follows gradual constriction of coronary arteries.³⁶ The synthesis of collagen associated with small vessels might be similarly enhanced.

Another mechanism that could lead to increased collagen synthesis during cardiac hypertrophy is that of scarring following cardiac necrosis. No light-microscopic evidence for necrosis, following aortic constriction, was observed in rat heart,³⁷ although occasional infarcts were noted in rabbit heart.³⁸ A statistical analysis of the spatial distribution of connective tissue nuclei, however, demonstrated that the nuclei were arranged more irregularly after than before hypertrophy. This observation was compatible with the possibility that connective tissue cells developed in areas of scattered necrosis.⁴ By electron microscopy, scattered cell necrosis and degeneration of myofibrils were noted during the first few days after aortic constriction.⁶ Hatt et al.³⁹ described dislocation of Z-bands, fragmentation of myofibrils, and disappearance of actin filaments in rabbit hearts that had failed acutely following marked stenosis of the ascending aorta. In an alternate interpretation of these morphological changes, Bishop and Cole⁴⁰ suggested that the Z-band was altered by a newly developing sarcomere. Thus, there is disagreement regarding the interpretation of anatomical evidence for cardiac necrosis following experimental production of aortic stenosis.

Biochemical studies have failed to demonstrate changes in myocardial protein metabolism in the hypertrophic heart that are consistent with extensive myofibrillar damage. The turnover of the total myocardial protein was unchanged during hypertrophy.⁴¹ The rate of degradation of myosin was not altered by the gradual development of hypertrophy,²⁸ and was actually decreased in more acute hypertrophy.⁴² Our observation that the peak of collagen labeling precedes that of

nuclear DNA labeling suggests that, during hypertrophy, collagen synthesis does not occur initially in new connective tissue cells that have replaced damaged muscle cells.

At least four combinations of changes in heart size and tissue collagen have been observed, depending upon the experimental procedure used.

1) Cardiac hypertrophy accompanied by an increase in tissue collagen concentration has been observed following repeated injections of isoproterenol to 300 g rats for 15 days, following constriction of the ascending aorta in 200 g rats⁴ (see Figure 5), 20-50 days following constriction of the main pulmonary artery of the adult cat,¹⁰ five months after constriction of the abdominal aorta of the weanling rat,⁴³ and six weeks after the onset of exposure of rats 2-1/2 months old to physical stress (although no changes were observed in rats which were 8 months old when stress adaptation was begun).⁴³ Crane and Dutta¹ observed that treatment of unilaterally nephrectomized rats with deoxycorticosterone resulted in cardiac hypertrophy accompanied by proliferation of connective tissue cells, but did not measure heart collagen.

2) Cardiac hypertrophy unaccompanied by a change in collagen concentration (i.e., the collagen increased in proportion to the heart enlargement) was observed in autopsy specimens of human hearts with left ventricular hypertrophy secondary to coronary atherosclerosis, rheumatic heart disease, renal disease, and chronic lung disease.⁴⁴ Oken and Boucek⁴⁵ observed similar changes in 12 of 14 human hearts enlarged as a result of coronary atherosclerosis; in two of these hearts, however, the collagen concentration was increased in association with cardiac hypertrophy.

3) Cardiac hypertrophy with no change in heart collagen content (and therefore no decrease in collagen concentration) was observed after 15 days of administration of thyroxine to 200 g rats,⁴³ and in rats 3-10 weeks of age that had developed sideropenic anemia secondary to dietary deficiency.⁴³

4) Increased collagen concentration unaccompanied by cardiac hypertrophy was observed after long-term (320 days) exposure of 200 g rats to a simulated altitude of 5000 m.⁴³

The collagen produced during the latter phase of hypertrophy could alter heart function. The property of preventing distension of blood vessels has been attributed to collagen.³³ Excessive distension of the heart and the resultant inefficient muscle contraction might be similarly prevented. On the other hand, it is possible that an increased amount of collagen in the hypertrophied heart might interfere with muscle contraction. Decreased contractility has been observed in papillary muscles of hypertrophied cat hearts.⁴⁶ It is possible to produce myocardial hypertrophy with or without increased collagen. A comparison of the mechanical properties of such preparations might help to elucidate the role of collagen in altering heart muscle function.

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INCREASED TURNOVER OF MITOCHONDRIAL CONSTITUENTS IN CARDIAC HYPERTROPHY AND ACUTE HYPOXIA IN THE RAT*

By

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Under normal conditions, cardiac muscle derives its energy almost exclusively from oxidative metabolism localized in mitochondria. Since the mitochondrion is the principal site of myocardial ATP synthesis, changes in mitochondrial functional mass may have important consequences for the metabolism of the rest of the cell. We now know that mitochondria are semi-autonomous organelles that have independent systems for the synthesis of nucleic acids and protein.¹⁻⁴ Heart mitochondrial components turn over rapidly with a half-life of about 5-6 days,⁵ indicating a capacity for rapid synthesis. In these experiments, we sought to study the effects of experimentally produced cardiac hypertrophy and of severe hypoxia on the synthesis and turnover of mitochondria and of other cardiac muscle components. Mitochondria are particularly suitable for these studies, since they are predominantly (over 95 per cent) localized in the cardiac muscle cell in whose function we are most interested. Furthermore, as the tissue receptors for oxygen, mitochondria may be particularly sensitive to changes in tissue oxygen tension or to levels of oxygen consumption.

Experimental cardiac hypertrophy was produced by banding of the ascending aorta in rats.⁶ As seen in Figure 1, a 20-40 per cent increase in heart weight is achieved within 36-48 hours after aortic constriction.

Changes in mitochondrial mass after aortic constriction were estimated from the activity of mitochondrial respiratory enzymes in homogenate and mitochondria. As seen in Figure 2, mitochondrial mass appears to increase proportionally more than total myocardial proteins 1 day after aortic banding, as indicated by the increase in homogenate specific activities of cytochrome oxidase, succinate cytochrome *c* reductase, and succinic dehydrogenase. Total cardiac activities of these enzymes increase to a greater extent than does total cardiac protein content. Mitochondrial enzymes therefore appear to be synthesized more rapidly than other protein components in early hypertrophy. This preferential synthesis of mitochondrial proteins is less apparent 3 days after banding.

Preferential synthesis of mitochondrial proteins in early cardiac hypertrophy is also indi-

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Figure 1. Changes in heart weight after banding the ascending aorta of rats. From Nair et al.⁶

Figure 2. Effect of banding the ascending aorta of rats on the cardiac respiratory enzyme activities. From Rabinowitz et al.⁷

cated by the relative incorporation of [³H]leucine into various myocardial subcellular fractions. Table 1 shows that, 24 hours after aortic banding, the increment in incorporation of [³H]leucine into mitochondrial proteins was greater than that into myofibrillar or supernatant proteins. It

Table 1
RELATIVE INCORPORATION OF [^{14}C]AMINO ACIDS INTO PROTEIN
OF SUBCELLULAR FRACTIONS OF HEARTS OF BANDED
AND CONTROL ANIMALS

Fraction	Sham	Banded	Ratio : $\frac{\text{Band}}{\text{Sham}} \times 100$
Homogenate	1.0	1.0	
Mitochondria	0.95	1.24	130.5
Myofibrils	0.97	0.90	93.0
Supernatant	1.28	1.35	105.5

may be concluded that mitochondrial proteins are synthesized rapidly during the acute phase of cardiac hypertrophy.

To gain insight into the dynamics of synthesis and degradation of mitochondrial proteins, we studied the effects of two experimental procedures: (a) cardiac hypertrophy produced by aortic constriction, and (b) exposure to severe hypoxia, on the turnover of mitochondrial proteins. We shall first discuss the effect of hypoxia and shall later return to cardiac hypertrophy.

The plan of the experiments to be described was to label mitochondrial protein or a specific mitochondrial cytochrome, several days before the experimental intervention. The effects of the experimental procedure on the specific radioactivity compared with controls reflects increased synthesis of new unlabeled material. Changes in the rate of destruction are measured by changes in the total cardiac radioactivity of a given component.

In the experiment shown in Figure 3, mitochondrial proteins were prelabeled with [^{14}C]-guanido-arginine. The rapid normal turnover of mitochondrial proteins is apparent. After exposure of rats to a 6-hour period of 4-5 per cent hypoxia, the specific activity of mitochondrial proteins was 15-20 per cent less than controls. This decrease is indicative of synthesis of new unlabeled mitochondrial proteins, presumably secondary to increased destruction produced by hypoxia. The slope of the decay curves for hypoxic and control animals is substantially the same, indicating that mitochondria that escape destruction during the hypoxic period are not more susceptible to destruction later.

These conclusions are confirmed by experiments using cytochrome c labeled with [^3H] δ -aminolevulinic acid, which is a non-reutilizable specific heme precursor. Accurate measurements of total cytochrome c content can also be made, which facilitate interpretation of the results. Cytochrome c is known to be bound to the inner mitochondrial membrane, and to turn over at the same rate as the particulate cytochromes b and aa₃.^{5,6,9}

The specific activity of cytochrome c (Figure 4) and also of isolated purified heme aa₃ decreased by 15-30 per cent after exposure to a low-oxygen atmosphere. No dilution was present at the end of the 6-hour hypoxic period, but it was present 6-12 hours after return to a normal atmosphere. This indicates that mitochondria may be destroyed during hypoxia, but that they are resynthesized only after return to a normal oxygen atmosphere. Also seen in Figure 4 is that hypoxia causes no decrease in myosin specific radioactivity. This indicates that mitochondria are more sensitive to the effects of hypoxia than is myosin.

Hypoxia leads to a significant decrease in cytochrome c content by the end of the 6-hour pe-

Effect of Hypoxia on The Turnover of Rat Heart Mitochondrial Proteins Labeled With [^{14}C] Arginine

Figure 3. Effect of a 6-hour period of 4-5% O_2 on the specific activities of mitochondrial protein previously labeled with [^{14}C]guanido-arginine. From Rabinowitz et al.⁷ and Aschenbrenner et al.⁸

Change in Specific Activity After Hypoxia Treatment

Figure 4. Effect of a 6-hour period of 4-5% O_2 on the specific activities of cytochrome c , and heme a prelabeled with [^3H] δ -aminolevulinic acid, and myosin prelabeled with [^3H]leucine. C = control; H + O = end of 6 hours of hypoxia; H + 6 = 6 hours after return to normal O_2 tensions. From Aschenbrenner et al.⁸

riod of hypoxia (Figure 5). The level returns to normal 6 hours later, indicating a brisk resynthesis. Thus, it seems clear that hypoxia results in a destruction of mitochondrial components that are rapidly synthesized after termination of the hypoxia.

Figure 5. Cardiac cytochrome c content after a 6-hour period of hypoxia, and 12 and 18 hours after return to a normal O_2 atmosphere. From Aschenbrenner et al.⁸

That mitochondrial resynthesis occurs predominantly after return to normal oxygen tension is confirmed by experiments measuring the incorporation of [3H]leucine into mitochondrial protein during and following hypoxia. Incorporation was at control levels during hypoxia, but increased 200-300 per cent 2 to 3 hours after return to normal oxygen tensions (Figure 6). The level of incorporation returns to normal by 24 hours.

The effect of aortic constriction on cytochrome c turnover was also studied. As seen in Figure 7, cytochrome c content increases by about 10 per cent following aortic banding. After correction is made for the increase in cytochrome c content, a 15-20 per cent dilution of cytochrome c specific activity is present. The decrease in specific radioactivity, and in total cardiac cytochrome c radioactivity compared with controls, indicates an increased turnover immediately after banding. The observed decrease in corrected specific radioactivity was unchanged 3 days after banding, indicating that the rate of destruction had returned toward normal.

From these data and from the turnover rate of cytochrome c, estimations of the rate of destruction and synthesis of cytochrome c can be made. It appears that the rate of destruction has increased considerably during the first 24 hours after banding. This increased rate of destruction is compensated by a threefold increase in the rate of synthesis. The rates of destruction and synthesis have returned toward normal 3 days after banding.

Figure 6. Incorporation of 1-hour pulses of [^3H]leucine into mitochondrial protein during the 6-hour period of 4-5% O_2 and after return to a normal atmosphere. From Aschenbrenner et al.⁸

Effect of Aortic Banding on Cytochrome c Content and Specific Radioactivity

Figure 7. Effect of banding of the ascending aorta of rats on total cardiac cytochrome c content, and on cytochrome c specific radioactivity, corrected for changes in cytochrome content. Cytochrome c was prelabeled with [^3H]δ-aminolevulinic acid 3 days prior to aortic banding. From Albin and Rabinowitz.¹⁰

CONCLUSION

Twenty-four hours after the production of cardiac hypertrophy in rats, the increase in mitochondrial mass is proportionately greater than that of other cardiac muscle proteins. It was also found that there is an increased rate of mitochondrial destruction 24 hours after aortic banding, and that this rate returns toward normal thereafter.

The experimental results show that severe hypoxia leads to mitochondrial destruction, but that mitochondrial components are rapidly resynthesized after a return to normal oxygen tension.

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T4 TRANSFER RNAs: CODON RECOGNITION AND TRANSLATIONAL PROPERTIES*

By

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Reports from this and other laboratories have described the occurrence of new aminoacyl-accepting tRNAs in phage-infected cells.¹⁻³ Based on the ability to hybridize to phage DNA, we have provided evidence for the presence of five tRNAs coded by the T4 genome (leucine, glycine, arginine, proline, and isoleucine), and a minimum of 14 phage tRNAs in T5-infected cells.⁴ The function of these tRNAs in phage replication is unknown. This study was undertaken to determine which code words, if any, are recognized by T4 tRNAs, and whether or not the phage tRNAs participate in polypeptide synthesis.

METHODS

Preparation of T4 tRNAs. Nitrocellulose filters (90 mm in diameter and impregnated with 1.5 mg of denatured T4 DNA per filter) were annealed with tRNA (2.4 mg tRNA per 6 mg of DNA) isolated from *Escherichia coli* B infected with T4 am 61, a lysozyme mutant, as previously reported.⁴ Each annealing reaction contained four filters in 15 ml of 50 per cent formamide-2 x SSC (0.15 M NaCP - 0.015 M Na citrate) (pH 6), and was incubated at 37° for 6 hours. After annealing, the filters were washed,¹ the nucleic acid was eluted from the filters by heating at 52° for 45 min. in 50 per cent formamide (50 ml for 10 filters), and the eluate dialyzed against 0.1 mM magnesium acetate. The dialyzed material was passed over a 3 cm x 0.5 cm DEAE-cellulose column and washed with water, followed by elution with 1 M NaCl-5 mM sodium phosphate (pH 6.3). The eluate that contained the tRNA was dialyzed and concentrated by lyophilization.

Ribosome binding assay. The tRNA-triplet binding assay was performed essentially as reported by Kan, Nirenberg, and Sueoka.⁵ Each 105 μ l reaction contained 5 μ M Tris·HCl (pH 7.3), 5 μ M of potassium acetate, 2 μ M of magnesium acetate, about 0.10 A₂₆₀ units of one oligonucleotide, and 1.0 A₂₆₀ unit of *E. coli* B ribosomes washed twice with high salt.⁶ Binding was initiated by the addition of the appropriate [³H]aminoacyl-tRNA as indicated in the text. After incubation for 15 minutes at 24°, the reaction mixture was diluted 20-fold with a cold solution containing 0.05 M Tris·HCl (pH 7.3), 0.005 M NaCl and 0.02 M magnesium acetate, and filtered (one drop per second) through Millipore discs. Prior to filtration, 500 μ g of yeast tRNA was added.

Charging of tRNAs. The reaction system used for amino-acid acylation of tRNA was identical to that described in Table 3 of a previous report.⁴ The synthetase enzyme used was treated with DEAE-cellulose.⁴ After reaction, the mixture was dialyzed, with one change, against 5 mM NaCl at 0° and used directly in the binding assays described in the text.

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Preparation of oligonucleotides. Nucleotide triplets were prepared enzymatically from oligonucleotide monophosphates⁷ and were also the generous gift from Dr. C. T. Caskey, Baylor University, Houston, Texas. The trinucleotide UUA was prepared by selective deamination of commercial CCA.⁸

The template RNAs from *E. coli*, T4-infected cells, and MS2 phage were prepared as described.⁶ [³H]amino acids were purchased from Mann-Schwarz, Orangeburg, N. Y., and New England Nuclear Corp., Boston, Mass.

RESULTS

Phage tRNAs, uncontaminated with host nucleic acid, were prepared by recovery of phage tRNA from its hybridization complex with phage DNA by a procedure partially modified from that described.⁴ Table 1 illustrates the absence of contamination of host tRNA, in the present scaled-up procedure, for several amino acids. Only those tRNA species previously established as being of phage origin showed aminoacyl acceptance activity.

Table 1
AMINO-ACID CHARGING OF TRANSFER RNA ISOLATED BY
HYBRIDIZATION TO T4 DNA

Radioactive amino acid	Specific activity (Ci/mM)	(A) Control: no RNA (cpm)	(B) T4 tRNA (cpm)	Difference (B - A) (cpm)
Leucine	50.0	1300	3660	2360
Proline	17.5	351	1878	1527
Glycine	30.0	735	4306	3571
Lysine	50.0	487	415	0
Valine	16.4	446	414	0
Phenylalanine	40.0	1170	1151	0
Alanine	35.0	270	305	30

Phage RNA from *E. coli* B infected with T4 am 61 was isolated by hybridization to T4 DNA as described under Methods. The presence of tRNA in this preparation was determined by charging with several different [³H]amino acids as shown above. The formation of [³H]aminoacyl-tRNAs was assayed by the appearance of acid-precipitable radioactivity.⁴ Control reactions contained no added RNA.

Assignment of codons for the amino acids studied was based on the stimulation of binding of radioactive aminoacyl-tRNAs to ribosomes in the presence of either trinucleotides of known sequence or synthetic ribopolymers. Tables 2 through 5 show comparisons of the ribosomal binding of *E. coli* and T4 tRNAs, charged with different radioactive amino acids, in the absence and presence of chemically defined oligonucleotides. Examination of the results reveals that the two different sources of tRNA exhibit distinct preferences in codon recognition for the same amino acid.

Table 2 shows that in the ribosome-binding assay *E. coli* glycyl-tRNA was stimulated substantially by GGU and GGC, but considerably less by the poly(G) oligomer. On the other hand, T4 glycyl-tRNA was stimulated most by poly(G), less by GGU, and only slightly by GGC.

Table 2

THE BINDING OF E. COLI AND T4 GLYCYL-tRNA TO RIBOSOMES
IN THE PRESENCE OF OLIGONUCLEOTIDES

Exp. no.	Oligonucleotide	<u>E. coli</u> [³ H]gly-tRNA (cpm)			T4 [³ H]gly-tRNA (cpm)		
		Input	Bound	Bound - control	Input	Bound	Bound - control
1.	None	5200	528		5200	690	
		10400	842		10400	1160	
	GGU	10400	4375	3847	10400	1910	750
	GGGG	5200	1020	492	5200	1505	815
		10400	1758	914	10400	2998	1838
2.	None	1600	60		1600	65	
		3200	108		3200	101	
	GGC	1600	580	520	1600	118	53
		3200	1100	992	3200	191	90

The binding assay was performed as described under Methods. E. coli and T4 tRNAs were charged with [³H]glycine (30 Ci/mM) and used in the binding reactions shown above, where indicated. The control counts are those obtained in the absence of any oligonucleotide. The assigned codons for glycine are GGU, GGC, GGA, and GGG.

Binding with E. coli arginyl-tRNA was strongest with the triplets CGU and CGA, and relatively weak with AGA and AGG (Table 3). The reverse was found for T4 arginyl-tRNA, however; binding was stimulated by AGA and AGG, but little or not at all by the cytidine-containing trinucleotides.

Of the two isoleucine codons examined in the binding assay, the E. coli tRNA species was preferentially stimulated by AUU, while T4 isoleucyl-tRNA preferred AUA (Table 4).

Table 5 indicates that, whereas the binding of E. coli leucyl-tRNA to ribosomes appeared to be stimulated by all of the established codons for leucine, significant binding for T4 leucyl-tRNA was observed only with the triplet UUA. This observation is in agreement with the codon prediction for T4 leucine-tRNA by examination of its primary nucleotide sequence as worked out by Pinkerton, Paddock, and Abelson.⁹

The degenerate nature of the code raises the question of specificity of a transfer RNA molecule for the recognition of one or more codons.¹⁰ With respect to phage tRNA function, a more immediate question is whether T4 tRNAs participate in polypeptide synthesis and, if so, whether they are employed for the translation of phage messengers. Preacylated E. coli and T4 tRNAs were compared in their ability to transfer labeled amino acids into a polypeptide form in the presence of different messengers. The in vitro, salt-washed ribosomal system was supplemented with initiation factors, supernatant factors, and the 19 complementary amino acids. The incorporation system was limited primarily by the amount of precharged tRNA present, but was also slightly limited in uncharged tRNA since addition of the latter showed small increments in protein labeling.

Table 6 indicates that, in the presence of different messenger RNAs, precharged T4 tRNAs transfer their esterified amino acids into hot acid-precipitable material as efficiently as pre-

Table 3

THE BINDING OF *E. COLI* AND T4 ARGINYL-tRNA TO RIBOSOMES
IN THE PRESENCE OF TRINUCLEOTIDES

Exp. no.	Trinucleotides	<i>E. coli</i> [³ H]arg-tRNA (cpm)			T4 [³ H]arg-tRNA (cpm)		
		Input	Bound	Bound - control	Input	Bound	Bound - control
1.	None	3000	118		3000	110	
		6000	240		6000	210	
	CGU	3000	1160	1042	3000	103	0
		6000	2470	2230	6000	206	0
	CGA	3000	880	762	3000	162	52
		6000	1876	1636	6000	347	137
2.	None	1625	85		1625	71	
		6500	280		3250	192	
	AGA	1625	120	35	1625	503	432
		6500	471	191	3250	1310	1118
	AGG	1625	125	40	1625	416	345
		6500	450	170	3250	991	799

The binding assay was as described under Methods. *E. coli* and T4 tRNAs were charged with [³H]arginine (35 Ci/mM) and used in the binding reactions shown above, where indicated. The assigned codons for arginine are CGU, CGC, CGA, AGA, and AGG.

charged *E. coli* tRNAs. An examination of the ratios of incorporation with T4 and *E. coli* messengers suggests that acylated T4 tRNAs are relatively more active with T4 mRNAs than with *E. coli* mRNAs. For two of the transfer RNAs, arginine and leucine, the incorporation ratios were two or more times higher with T4 tRNAs. The change in incorporation ratios is primarily

Table 4

THE BINDING OF *E. COLI* and T4 ISOLEUCYL-tRNA TO RIBOSOMES
IN THE PRESENCE OF TRINUCLEOTIDES

Trinucleotide	<i>E. coli</i> [³ H]ileu-tRNA (cpm)			T4 [³ H]ileu-tRNA (cpm)		
	Input	Bound	Bound - control	Input	Bound	Bound - control
None	1700	136		1530	152	
	3460	266		3060	250	
AUU	1700	348	212	1530	149	0
	3460	681	415	3060	277	27
AUA	1700	160	24	1530	183	31
	3460	270	4	3060	421	171

The binding assay was as described under Methods. *E. coli* and t4 tRNAs were charged with [³H]isoleucine (34 Ci/mM) and used in the binding reactions shown above, where indicated. The assigned codons for isoleucine are AUU, AUC, and AUA.

Table 5

THE BINDING OF *E. COLI* AND T4 LEUCYL-tRNA TO RIBOSOMES
IN THE PRESENCE OF DIFFERENT OLIGONUCLEOTIDES

Exp. no.	Oligonucleotide	<i>E. coli</i> [³ H]leu-tRNA (cpm)			T4 [³ H]leu-tRNA (cpm)		
		Input	Bound	Bound - control	Input	Bound	Bound - control
1.	None	2560	235		5120	1215	
		5120	467				
	UUG	2560	318	83	5120	1174	0
		5120	580	113			
	Poly(C,U) (1:2)	2560	537	302	5120	1195	0
		5120	1240	773			
	CUA	2560	265	30	5120	1208	0
		5120	580	113			
2.	CUG	2560	619	384	5120	1153	0
		5120	1313	846			
	None	5500	431		4800	1203	
	UUA	5500	565	134	4800	1425	222
3.	None	5500	515		4800	809	
	UUA	5500	482	0	4800	1116	307

The binding assay was as described under Methods. *E. coli* and T4 tRNAs were charged with [³H]leucine (50 Ci/mM) and used in the binding reactions shown above, where indicated. In Exp. 3, the reaction system contained half the amount of washed ribosomes (0.5 A₂₆₀ units) as in Exps. 1 and 2. The assigned codons for leucine are UUA, UUG, CUU, CUC, CUA, and CUG.

the result of a lower efficiency of *E. coli* mRNA translation with T4 aminoacyl-tRNAs. Except for arginyl-tRNA, the level of amino-acid incorporation with T4 mRNA was similar with both sources of tRNA. The fact that all of the phage tRNAs are relatively more active with T4 mRNA seems not to be coincidental. Table 6 also shows that a comparison of incorporation between T4 and MS2 RNA templates for both kinds of tRNAs results in a random preference, i.e., the T4:MS2 incorporation ratio remains the same for *E. coli* and T4 glycyl-tRNAs, decreases for T4 leucyl-tRNA, increases for T4 isoleucyl-tRNA, and shows no incorporation of arginine when T4 tRNA is used with MS2 RNA.

DISCUSSION

The results presented here indicate that, although phage tRNAs recognize the same codons previously assigned for a given amino acid, they tend to prefer code words that are less well recognized by their *E. coli* B host. Daniel, Sarid, and Littauer¹² had previously suggested that such a preference might exist. If these "T4-preferred" codons appear with greater frequency in T4 mRNA than in host messenger (as suggested by the results in Table 6), then the concentration of the appropriate host tRNAs might be too low for efficient read-out of T4 messengers. To avoid such a predicament, certain phages appear to have acquired sufficient (genetic) information to direct the synthesis of their own tRNA molecules. Nevertheless, *E. coli* B tRNAs do rec-

Table 6
COMPARISON OF THE USE OF T4 AND *E. COLI* AMINOACYL-tRNAs
IN POLYPEPTIDE SYNTHESIS

³ H]aminoacyl-tRNA			mRNA	Incorporation (cpm)	mRNA incorporation ratio	
Amino acid	tRNA source	Input (cpm)			T4/ <i>E. coli</i>	T4/MS2
Glycine	<i>E. coli</i>	3000	None	138	1.48	1.27
			T4	1078		
			<i>E. coli</i>	773		
			MS2	885		
	T4	3200	None	140	2.45	1.32
			T4	1111		
			<i>E. coli</i>	536		
			MS2	876		
Arginine	<i>E. coli</i>	2800	None	58	1.46	2.55
			T4	659		
			<i>E. coli</i>	469		
			MS2	294		
	T4	2800	None	55	2.72	
			T4	175		
			<i>E. coli</i>	99		
			MS2	46		
Leucine	<i>E. coli</i>	4760	None	134	1.77	4.85
			T4	1250		
			<i>E. coli</i>	763		
			MS2	364		
	T4	4640	None	180	4.57	2.76
			T4	1209		
			<i>E. coli</i>	405		
			MS2	553		
Isoleucine	<i>E. coli</i>	6800	None	213	1.50	1.44
			T4	2658		
			<i>E. coli</i>	1835		
			MS2	1918		
	T4	7200	None	518	1.82	2.69
			<i>E. coli</i>	2548		
			T4	1601		
			MS2	1273		

The reaction system was essentially the same as described by Klem et al,¹¹ except that no added tRNA was present. An S165 soluble supernatant fraction, derived from 1/4 log *E. coli* B cells, was treated with DEAE-cellulose to remove endogenous tRNA and dialyzed.¹⁴ 40 µg of protein from this treated S165 preparation were added to each reaction mixture as a source of synthetase and elongation factors. Transfer RNA charged with [³H]amino acids was introduced into the reaction mixture as shown above. Except for the labeled species, all other amino acids were present in the reaction at 0.01 mM. Protein synthesis was supported by tRNA present in the washed ribosomes (one A₂₆₀ unit), equivalent to approximately 1 µg per reaction as estimated by amino acid acceptance activity. Initiation factors⁶ were also added to give optimal incorporation. The amount of mRNA added was 100 µg T4 mRNA (isolated from 12-min. infected cells at 37°); 112 µg for *E. coli* B mRNA, and 22 µg for MS2 RNA. After incubation for 15 min. at 37°, the reaction was stopped with trichloroacetic acid, heated at 90°, filtered, and washed by the standard procedure for radioactivity determination.⁶ The incorporation ratios were calculated after subtraction of the appropriate washout control values (no mRNA).

ognize all of the codons recognized by T4 tRNAs (albeit less efficiently), suggesting that the T4 tRNAs should not be essential for T4 replication. Recent work by John Wilson¹³ indicates that T4 phage mutants that are negative with respect to phage tRNA synthesis replicate well in E. coli B, thus supporting the idea of a nonessential role played by the phage transfer RNAs. When strains of E. coli other than B were used as hosts, however, these same phage mutants showed no replication. These findings suggest that during the evolution of some bacteriophages, at least for T4, a mechanism has been provided to insure continued T4 replication in hosts whose biochemical machinery might not include a complete assortment of the necessary transfer RNAs for translation of phage messengers. It is interesting to speculate how phages acquired their tRNA genes, and why, during phage evolution, those tRNA molecules were acquired that recognize codons infrequently used by their host. Although several groups have demonstrated the in vitro synthesis of phage proteins (enzymes) in the absence of phage tRNAs,^{15,16} the possibility still exists that these tRNA molecules may serve some specific function (e.g., regulatory) during phage development.

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SMALL-ANGLE X-RAY SCATTERING OF HUMAN SERUM HIGH-DENSITY LIPOPROTEINS*

By

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The chemical compositions and many of the physical properties of the various lipoprotein classes obtained from human serum have been defined, but comparatively little is known about their structural organization and the modes of interaction between the major lipid and protein components.^{1,2} In the case of the high-density lipoproteins (HDL), significant advances have resulted from the preparation of a water-soluble, lipid-free apoprotein,³ its further fractionation into peptide subunits,^{4,5} and the recombination of both apoprotein and major peptides with lipids to give reconstituted lipoproteins.⁶⁻⁸

Recently, spectroscopic techniques including optical rotatory dispersion⁹ and circular dichroism,¹⁰ nuclear magnetic resonance,^{11,12} and electron spin resonance¹³ have been applied to native lipoproteins and, in the case of the high-density fraction HDL₂, to a reconstituted lipoprotein. Although these techniques are providing valuable information on the conformation, environment, and interactions of individual components, so far details of the overall structure have depended on the use of electron microscopy.¹⁴

Small-angle X-ray scattering (SAXRS), which has been used to probe the overall size and internal structure of micellar lipid solutions¹⁵ and some model lipoproteins,¹⁶ would seem to be a technique suited to the investigation of these structural features of serum lipoproteins. In this paper we describe the SAXRS from native human serum high-density lipoprotein (HDL₂). A similar approach to the study of reconstituted lipoproteins formed by the recombination of lipid-free water-soluble protein, apo-HDL₂, with various lipids will be reported later.

METHODS

Preparation of HDL₂. HDL₂ was separated by ultracentrifugal flotation from sera of healthy, human Caucasian donors (Type A, Rh⁺), as previously described,¹⁷ and dialyzed for 48 hours at 4° against several changes of 0.15 M NaCl, pH 7, containing 10⁻³ M EDTA.

Small-angle X-ray scattering. The X-ray scattering measurements were recorded using a Kratky camera,¹⁸ with divergent beam geometry, adapted to a highly stabilized Philips PW 1010 generator. The entrance slit width, determining the resolution of the camera, was normally 100μ, with a corresponding counter slit width of 217μ. The distance between the sample cell and the recording plane was either 205 mm or 215 mm. The scattered radiation was detected by a xenon-filled proportional counter and measured using a transistorized counting chain. Monochromatiza-

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tion of the copper radiation was achieved with a nickel β -filter in conjunction with pulse height discrimination.

HDL₂ samples were mounted in 1 mm-diameter, thin-walled glass capillary tubes, and the particle scattering curves were derived by subtraction of the solvent from the solution scattering curves. The scattering curves, corrected for the effect of the line collimation system ("desmearing"), were computed using the methods described by Taylor and Schmidt¹⁹ and Lake.²⁰

X-ray scattering curves from a concentrated HDL₂ solution (19.8 mg protein per ml) were obtained using Elliott toroidal optics²¹ and the high-intensity focal spot (0.2 mm²) of the Elliott GX 6 rotating-anode generator.

RESULTS AND DISCUSSION

The scattering curve I(h) from HDL₂ (8.5 mg protein per ml) in 0.15 M NaCl, 10⁻³ M EDTA buffer is shown in Figure 1(a). This curve has been obtained reproducibly from many samples of HDL₂ and, for low values of h, shows the usual decrease in scattered intensity with increasing h. More importantly, it shows two clearly defined maxima centered at $h = 0.075 \text{ \AA}^{-1}$ and

Figure 1. X-ray scattering curve of human serum HDL₂ (8.50 mg protein per ml) in 0.15 M NaCl, 10⁻³ M EDTA at pH 7. (a) Scattering curve I(h) containing smearing effect. Insert: Microdensitometer trace from X-ray photograph (see text) of HDL₂ (19.8 mg protein per ml). (b) Scattering curve as in (a), with the smearing effect removed.

$0.152 \text{ \AA}^{-1}$. The broad maxima in the region $h = 0.20$ to $0.40 \text{ \AA}^{-1}$ is probably due to further poorly resolved subsidiary maxima. This region of the scattering curve was further examined using photographic methods, and the microdensitometer trace [insert, Figure 1(a)], showing a maximum at $h = 0.225 \text{ \AA}^{-1}$, would appear to confirm the above interpretation.

The two methods used for desmearing the experimental curves show good agreement, although 30 iterations of the Lake procedure²⁰ were sometimes required to produce convergence. As shown in Figure 1(b), desmearing shifts the positions of the maxima, accentuates the minima

and, due to termination effects, slightly distorts the scattering curve in the region near the maximum value of h . This last effect probably accounts for the slight asymmetry of the two subsidiary maxima in the desmeared curve. In order to eliminate the effect of interparticle interference on the scattering curves, we recorded the inner parts of the curves at several HDL₂ concentrations in the range from 2.00 to 8.50 mg protein per ml.

According to the treatment of Guinier,²² the radius of gyration, R_g , is obtained from the initial slope of a plot of $\log I$ versus h^2 . Linear extrapolation to $h = 0$ gives the scattered intensity at zero angle (I_0), and R_g is determined from the relation

$$\log_e I = \log_e I_0 - 1/3 R_g^2 h^2, \text{ where } h = \frac{4\pi \sin \theta}{\lambda}.$$

"Guinier plots" of the smeared and desmeared scattering curves, producing $\tilde{R}_g$ and R_g , respectively, are linear at low values of h^2 . The Guinier plots of the desmeared scattering curves at three HDL₂ concentrations are shown in Figure 2. In Figure 3, the radii of gyration obtained

Figure 2. Guinier plots ($\log I$ vs. h^2) for human serum HDL₂. Δ , 2.0 mg protein per ml; $\circ$, 4.25 mg protein per ml; $\square$, 8.5 mg protein per ml.

from both the smeared and desmeared scattering curves are plotted as a function of concentration. We observe that desmearing has only a small effect on the radius of gyration, as may be anticipated from the extensive linear region of the Guinier plots. Furthermore, the linear dependence of the radius of gyration on concentration is typical of that resulting from interparticle interference. The radii of gyration extrapolated to zero concentration give $\tilde{R}_{gC=0} = 44 \text{ \AA}$ and $R_{gC=0} = 46 \text{ \AA}$ for the smeared and desmeared data, respectively.

Consideration of the resolved subsidiary maxima in the X-ray scattering curve, together with the extensive linear region shown in its Guinier representation, would suggest that HDL₂ is a highly symmetrical particle. Assuming a spherical shape for the particle and a uniform

Figure 3. Plot of radius of gyration as a function of concentration C (mg protein per ml). (a) R_g vs. C ; (b) $\tilde{R}_g$ vs. C ; scattering curve desmeared using $\circ$: Lake procedure, $\times$: Schmidt procedure.

electron density distribution, and using the zero-concentration radius of gyration ($R_{gC=0}$) of 46 Å, we calculate a particle diameter of 119 Å. With the same assumption regarding shape, we can calculate a molecular diameter by using a molecular weight of 386,000 obtained by sedimentation equilibrium ultracentrifugation and the partial specific volume 0.914 ml gm⁻¹. This procedure gives a molecular diameter of 104 Å. Both of these values for the diameter of HDL₂ exceed those obtained by electron microscopy of negative-stained preparations of HDL₂, which gave particle diameters in the region of 90-100 Å, assuming again that the particles viewed are spheres and not rods.¹⁴ Thus, if we make the assumption of sphericity, there is some discrepancy in the values for diameters of HDL₂ obtained by using these independent techniques.

A comparison of the scattering curves obtained from the X-ray data with the theoretical scattering curves for model globular particles (e.g., spheres, ellipsoids) of uniform electron density reveals that the positions and, more strikingly, the relative intensities of the subsidiary maxima, do not coincide with those expected for this type of particle. For example, the relative intensities of the subsidiary maxima of the experimental curve may exceed by a factor of ten those of the calculated curves.²³ This appears to confirm the view that the uniformly electron-dense, spherical model for HDL₂ is of doubtful validity.

An extension of this approach has involved the calculation of scattering curves for symmetrical particles with a nonuniform electron density distribution. In particular, a detailed study has been made of spherical particles with a radial electron density distribution within the particle, the distribution being defined by a four-parameter step model (see Figure 4). Theoretical scattering curves were calculated for spherical particles in which the external radius R , the internal radius γR , and a parameter β relating the electron densities of the core and shell were varied systematically while changes in the positions and relative intensities of the subsidiary maxima were monitored. Compared with the uniform-sphere model, enhancements of the relative intensities of the subsidiary maxima were obtained. Detailed comparison with the desmeared

Figure 4. Step model of spherical particle with radial electron density distribution.

HDL₂ experimental curve has led to the optimal agreement shown in Figure 5, the theoretical curve being calculated using the parameters $R = 108 \text{ Å}$, $\gamma = 0.80$, and $\beta = -0.263$. The agreement in terms of peak positions and peak intensities is excellent, although the peak widths are rather different and the minima in the theoretical curve are lower. Reasonable agreement is observed in the small-angle region of the scattering curves.

Figure 5. Comparison of experimental and theoretical X-ray scattering curves: —, desmeared experimental curve for HDL₂ (extrapolation to zero concentration); ----, theoretical scattering curve for spherical particle with radial electron density distribution ($R = 54 \text{ Å}$, $\gamma = 0.80$, $\beta = -0.263$).

This model with a relatively electron-deficient central core (diameter = 86 Å) surrounded by an electron-rich outer shell (thickness = 11 Å) is consistent with the evidence favoring a surface location for the protein subunits,²⁴ particularly the glutamic and lysine amino-acid residues, and the polar moieties of the constituent phospholipids^{12,25} of HDL₂. Also, it might be expected that the apolar parts of the molecular constituents, e.g., the hydrocarbon chains of the phospholipids and the cholesterol esters, might be located in the interior of the particle, this region being identified with the electron-deficient core in the step model. However, although this is undoubtedly the best description of a spherical HDL₂ particle, the fact that the minima in the scattering curves do not reach zero on the abscissa probably indicates that small deviations from ideal spherical symmetry do occur. Similar effects in the scattering curves of other highly symmetrical particles, for example, latex spheres,²⁶ plant viruses,²⁷ and macrophages,²⁸ have been reported. Thus, further work embodying calculations of the scattering curves for nonuniform, nonspherical particles, and eventually a Fourier inversion of the intensity distribution giving an electron density profile of the HDL₂ particle, is necessary before an unequivocal commitment to the present structure is made.

The detailed description of the structure of serum lipoproteins has so far depended upon the use of electron microscopy. The X-ray scattering study reported here shows that this technique is capable of providing a quantitative structural characterization (size, shape, internal structure) in the case of HDL₂, and indicates its applicability to other soluble, native and reconstituted lipoprotein systems.

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STABILIZATION OF URINARY ERYTHROPOIETIN*

By

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Urine from anemic patients has been an important source of large amounts of crude erythropoietin.¹ Plasma from anemic animals has yielded a preparation of erythropoietin that is virtually homogenous by gel electrophoresis.² Although a high potency fraction of the urinary hormone has been described,³ no evidence regarding its homogeneity has been published. One of the impediments in purifying urinary erythropoietin, despite the initial advantage of having starting material with a low protein concentration, is its marked instability. Graham et al⁴ have shown that there are large losses during fractionation, and in our early experience with urinary erythropoietin concentrates, losses were considerably greater than those we found for comparable steps when working with plasma. Loss of activity was even found during storage at -20°C.

We have determined that urine concentrates contain both protease and sialidase activities that probably are the cause of the instability. The method described in this communication makes it possible to prepare from urine crude fractions that are low in proteases and sialidase and are stable. There has been a previous report of protection of erythropoietin activity by use of a small amount of phenol as a bacteriocidal agent.⁵

MATERIALS AND METHODS

Starting materials were two different crude precipitates from erythropoietin-rich human urine. They were generously supplied by the National Blood Resources Branch of the National Heart and Lung Institute, Bethesda, Maryland, and were derived from the collection center in Corrientes, Argentina with some processing at the Children's Hospital, Los Angeles, California. One preparation was labeled C-1 and the other, TaLSL.

Assay for erythropoietin was done by the fasted-rat method,⁶ using as a standard a plasma fraction that had been standardized against the International Reference preparation. All assays were done with groups of five rats and by interpolation of the log dose-log response standard curve using standard doses of 1, 2, and 3 units per rat. Protein concentrations were determined by measurement of absorbance at 278 nm, and using the value of $A_{278}^{1\%} = 10$ derived from measurements of mixtures of glycoproteins.

Assay for protease activity was done using as substrate an insoluble collagen-dye complex (Azocoll, Calbiochem). A suspension of 1 mg of the substrate in 200 μ l of 0.1 M phosphate buffer at pH 7.0; mixed with 1 mg of crude urinary erythropoietin preparations in 200 μ l of the same buffer, was incubated for 15 min. at 37°; the insoluble material was removed and the solution

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absorbance at 530 nm was read. A blank consisting of the same concentration of substrate, but with no source of protease, was incubated simultaneously and the activity determined from the difference between the blank and experimental readings.

Sialidase activity was determined using sialyllactose (5 mg/ml in 0.1 M Na acetate, 0.01 M CaCl_2 , pH 4.5) as substrate. After incubation with the erythropoietin preparations for 3 hours, the free sialic acid was determined by the alkaline Ehrlich's method.⁷

Treatment of erythropoietin samples. The C-1 material, at 10 mg/ml, was dissolved in 8 M urea; the urea was then removed by pressure ultrafiltration through a UM-10 membrane (Amicon Corporation, Lexington, Massachusetts) in the cold. This preparation is so insoluble that a high concentration of urea is needed to solubilize it; the activity then stays in solution when the urea is removed. After complete removal of urea, the crude erythropoietin in phosphate-buffered saline (PBS) was heated for 5 min. at 100°C. The solution was chilled and centrifuged, and the precipitate was washed once with cold PBS. The pooled supernatants were frozen until the next step.

Because it is more easily dissolved, the TaLSL preparation was put directly into cold PBS (10 mg/ml), then heated at 100°C for one min. and centrifuged as described for the C-1 preparation.

A mixture of one volume of PBS, one volume of 0.2 M phosphate buffer at pH 7.0, three volumes of 0.475 sodium p-aminosalicylate (PAS), and 5 volumes of freshly distilled phenol was shaken vigorously in the cold, and after phase separation the upper (aqueous) phase was discarded. The lower phase, phenol-equilibrated with buffer and PAS, was used to treat the erythropoietin preparations.

To each preparation in PBS, enough phosphate buffer at pH 7.0 and PAS were added to make the final concentrations 0.04 M and 0.285 M, respectively. They were then shaken vigorously with an equal volume of phenol and the phases were separated by centrifugation in the cold. The separated fractions (including any interphase material) were dialyzed against PBS in the cold to remove phenol, and concentrated by pressure ultrafiltration. The fraction derived from the phenol phase still contained an appreciable amount of phenol, which was removed by precipitation of the proteins with cold alcohol at a final concentration of 90 per cent. The precipitate was dissolved in PBS before assay.

Table 1
PROTEASE AND SIALIDASE ACTIVITIES IN
ERYTHROPOIETIN PREPARATIONS

Sample	Protease activity	Sialidase activity
	ΔA_{530}^*	Sialic acid freed (μg) in 180 min.
Original C-1	0.057	0.18
Phenol-treated C-1	0.013	0.0
Alcohol-precipitated, phenol-treated C-1	0.015	0.0

* ΔA_{530} represents the difference between the blank and the experimental absorbance.

Assays for protease and sialidase activities in the treated and untreated erythropoietin preparations are shown in Table 1. After the phenol extraction about 75 per cent of the protease activity was lost; alcohol precipitation did not cause any further loss. The inactivation of sialidase activity in the treated preparations was complete.

RESULTS

Results of bioassay of fractions from both erythropoietin preparations are presented in Table 2. The data show that more than 90 per cent of the activity was recovered from the phenol phase with a small increase in potency. When the phenol extraction was done in the absence of

Table 2

ERYTHROPOIETIN ACTIVITY OF PHENOL-TREATED FRACTIONS*

Sample	Total units	Potency (units/mg)	Recovery (%)
Heated C-1	3820	7.5	(100)
Upper and interphases	0		0
Lower phase	3660		95.8
Alcohol precipitate	3540	10.5	92.7
Heated TaLSL	7220	32	(100)
Upper and interphases	0		0
Lower phase	7070		97.9
Alcohol precipitate	6500	47	90.0

* These are the average results of six experiments using C-1 and three using TaLSL.

Figure 1. Stabilization of urinary erythropoietin by phenol treatment.
o = Untreated; x = Phenol treated.

PAS, activity could be variably recovered in all three fractions. The stability of the phenol-treated C-1 fraction is compared with that of the heat-treated extract in Figure 1. For at least the first 16 weeks of storage in the frozen state, the phenol-treated erythropoietin concentrate lost very little activity, while the loss of activity from the starting material was substantial.

The use of this method of stabilization should permit the development of a more efficient procedure for the isolation of pure human erythropoietin.

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USE OF [³H]-THYMIDINE FOR MEASUREMENT OF DNA SYNTHESIS IN RAT LIVER—A WARNING*

By

C. G. D. Morley and H. S. Kingdon

Many methods for the extraction of DNA from both plant and animal tissues have been published; those most often used were devised by Schmidt and Thannhauser¹ and Schneider.² The methods involve precipitation of proteins and nucleic acids from the tissues by treatment with ice-cold TCA (or perchloric acid), followed by lipid removal with washes of the precipitated material with alcohol and ether, and finally extraction of DNA with hot 10 per cent TCA (or perchloric acid). This procedure has been used by many investigators studying DNA synthesis using radioisotopes such as [³H]-thymidine. We have also attempted to use the method in our studies of [³H]-thymidine uptake by hepatocytes as a measure of DNA synthesis. A major difficulty was encountered, however, because the use of hot acid in the final step of the procedure resulted in the extraction of a considerable amount of radioactive label not associated with DNA. This problem has not previously been mentioned, and we considered it would be of some importance to warn other workers using these techniques, particularly with hepatocytes, of the problems that may be encountered, and to detail ways of avoiding them. This paper, therefore, reports (a) the extent and possible character of the nonspecific binding agent(s)[†] for [³H]-thymidine in hepatocytes, (b) a modification of an accepted DNA extraction procedure which essentially overcomes the difficulty of nonspecific binding of [³H]-thymidine, and (c) a suitable control system to allow for this nonspecific binding when using the acid precipitation extraction (or any other DNA extraction) procedure.

MATERIALS AND METHODS

Livers were excised from male Sprague-Dawley albino rats (300-400 g), and chilled in ice-cold 0.85 per cent NaCl. They were then minced with scissors, washed thoroughly with ice-cold saline, and pushed through three stainless-steel sieves (Dual Manufacturing Co., Chicago, Ill., sizes 420 μ , 250 μ , and 177 μ),³ using gloved fingers. The cells were washed through the sieves with cold saline. The resulting single-cell suspension was spun for 5 min. at 5000 rpm at 2° and the supernatant solution was discarded. The cell pellet was resuspended in cold 0.85 per cent saline by gently working the cells through a 17-gauge hypodermic needle.

Partial hepatectomies were performed under ether anesthesia according to the method of Higgins and Anderson.⁴

[³H-Methyl]-thymidine (specific activity 8.7 Ci/mmole) was purchased from International Chemical and Nuclear Corp., Irvine, Calif. Trypsin, chymotrypsin, and α -amylase were obtained from Nutritional Biochemicals.

For routine measurement of DNA synthesis, approximately 1×10^6 cells were incubated at

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† The term "nonspecific binding" refers in this paper to any binding of tritiated thymidine not directly connected with DNA.

37° in Difco, minimal Eagle's medium (Spinner modified for suspension cultures, No. 5838) containing 1000 units of penicillin and 1000 µg of streptomycin per milliliter. [³H-Methyl]-thymidine (8 x 10⁶ cpm) was added to the suspension (final volume 2 ml) in 10 ml polyethylene tubes. In general, the incubation periods were from 15 min. to 1 hour for these experiments. After incubation, the cells were quickly spun down at 10,000 rpm and 2° for 5 min., washed twice by resuspension on a vortex mixer in 2 ml of 0.05 M potassium EDTA, pH 7.0 (ice-cold), and then assayed for radioactive label incorporated by either the "acid" technique or the "phenol" method (both described below).

Liquid scintillation counting was performed in a Packard Tri-Carb Model 3375 liquid scintillation counter using 10 ml of Aquasol fluid (Nuclear-Chicago Corp.).

"Acid precipitation" technique for DNA extraction. The method used in these experiments is modified from both Schmidt and Thannhauser¹ and Schneider.² Following two washes with EDTA as above, the cell pellet was treated with ice-cold 10 per cent TCA (2 ml), shaken on a vortex mixer, and spun briefly in a bench centrifuge. The supernatant solution was discarded and the pellet was washed three times more with acid, followed by three washes with room-temperature alcohol (2 ml) and once more with cold acid (2 ml). DNA was then extracted from the residue with 10 per cent TCA (2 ml) at 90° for 15 min. After centrifugation, aliquots of the supernatant solution were taken for radioactive counting and total DNA estimation by a modification of the method of Dische^{5,6} and by the indole method.⁷ Specific radioactivities of DNA were calculated as counts per minute per milligram of DNA.

RESULTS AND DISCUSSION

Nonspecific binding of [³H-methyl]-thymidine by hepatocytes. Table 1 shows the results of incubating liver cells with tritiated thymidine under conditions in which no DNA is synthesized. All three samples—boiled cells, TCA-precipitated cells, and frozen cells, none of which could

Table 1
NON-DNA ASSOCIATED [³H]-THYMIDINE*

Treatment [†]	Total cpm	Specific activity, cpm/mg DNA
(1) Boiled cells	1116	2.3 x 10 ^{5†}
(2) TCA cells	1013	2.1 x 10 ^{5†}
(3) Frozen cells	451	1.0 x 10 ^{5†}
(4) Normal cells	600	1.5 x 10 ⁵

* Cells were incubated for 30 min. at 37° as described in the text and assayed for DNA synthesis by the acid precipitation method.

[†] Treatments were as follows: (1) Cells were boiled for 2 min. at 100° prior to incubation with [³H-methyl]-thymidine; (2) cells were pretreated with 10% TCA (ice-cold), washed with ice-cold water until the pH was approximately 6.0, and then incubated with [³H-methyl]-thymidine; (3) cells were quick-frozen, after addition of [³H-methyl]-thymidine, in acetone-dry ice mixture and kept frozen during the incubation period.

[‡] Specific activity for these samples is not really a DNA specific activity since no DNA was synthesized in these samples. However, in order to keep the tables consistent the specific activities are given as for DNA (see also Footnote † in Table 5).

have synthesized DNA—nevertheless incorporated significant amounts of radioactivity. In fact, two of the samples showed higher "specific activities" for their DNA than the control cells that did synthesize DNA.

In order to be sure that the tritium incorporated by samples 1 to 3 in Table 1 was not associated with DNA, samples of liver cell extract (obtained by sonication of liver cells at 0° for 30 sec. in Difco medium) free of DNA (checked by the colorimetric assays) were incubated under the usual conditions with tritiated thymidine. The samples were then assayed by the "acid" technique to determine incorporation of label into extractable material. Table 2 shows the results of such an experiment in which the samples of cell extract were treated, prior to addition of tritiated thymidine, with α -amylase (to remove glycogen), trypsin, and chymotrypsin. This was done in an attempt to identify the type of molecule causing the nonspecific binding of thymidine.

Table 2

[³H-METHYL]-THYMIDINE BINDING TO PROTEINS IN LIVER CELL EXTRACTS*

Preincubation with	DNA (μ g)	Protein (peptide) (mg)	Cpm isolated by TCA precipitation method
Nothing	0 [†]	6 [§]	2784
Albumin [†]	0	7	3117
α -Amylase [†]	0	5	1480
Trypsin [†]	0	4	238
Chymotrypsin [†]	0	2.5	236

* 2 ml of cell extract (72 mg of protein), prepared as described in the text, were incubated for 30 min. at 37° with [³H-methyl]-thymidine under the usual assay conditions.

[†] Preincubations were with 500 μ g of the appropriate protein at 37° for 60 min.

[‡] Zero indicates not detectable by either indole method or the diphenylamine method, showing that, if any, less than 1 μ g was present in the cell extract.

[§] Measured by the biuret method.

The results in Table 2 show that considerable amounts of radioactive label were bound by the cell extract, none of which could have been associated with DNA (see above). Treatment of the extract with α -amylase decreased the binding of thymidine by about 50 per cent (although, as discussed below, this is probably not due to removal of glycogen), but either trypsin or chymotrypsin removed over 90 per cent of the material responsible for the thymidine binding in the cell extract. (The 50 per cent loss of binding capacity with amylase treatment is probably due to protease contamination of the amylase preparation.)

Table 2 indicates that the non-DNA bound-tritiated thymidine in hepatocytes is located on protease-sensitive substances within the cells. To confirm this point, the uptake of tritiated thymidine by whole cells was compared with that of whole cells treated with an inhibitor of DNA synthesis (see next section), and with that of cell extract obtained from an equivalent number of whole cells. Table 3 shows the results of this experiment, which clearly indicates that the non-DNA associated radioactive label is bound to unknown proteins in the hepatocytes. It is interesting to note that the amount of label bound by the cell extract is 50 per cent greater than that bound in the cells treated with the inhibitor of DNA synthesis (hydroxyurea). Presumably this is

Table 3

NON-DNA ASSOCIATED [³H-METHYL]-THYMIDINE EXTRACTED FROM
CELL EXTRACTS AND WHOLE LIVER CELLS*

Sample	Treatment	Counts per minute
(1) Whole cells	Hydroxyurea [†]	351
(2) Whole cells	None	1027
(3) Cell extract [‡]	None	620

*Incubations were as described in the text, using 2×10^6 cells.

[†]25 mM hydroxyurea in 0.85% NaCl solution was added prior to incubation with [³H-methyl]-thymidine.

[‡]Cell extract was prepared as described in the text from 2×10^6 cells, to be comparable with experiment (1).

due to the more intimate contact of the proteins of the cell extract with the labeled nucleoside.

Use of hydroxyurea in control samples to allow for "protein bound" thymidine. The use of a convenient inhibitor of DNA synthesis in the whole cells allows us to make adjustments for any nonspecific binding of thymidine in the cell. As shown in Table 3, hydroxyurea is a useful inhibitor⁸ which, at 25 mM concentration, is capable of stopping DNA synthesis in the hepatocyte.

Table 4 shows the kind of result that can be expected with cells from either normal or regenerating liver, 23 hours after partial hepatectomy, using hydroxyurea as a control and the acid extraction assay. Slightly better inhibition is obtained (especially for short incubations with triated thymidine) if the cells are preincubated with hydroxyurea for 15 to 30 min.

Table 4

USE OF HYDROXYUREA AS CONTROL

Sample	Experiment number	Treatment	Incubation time (min.)	Specific activity of DNA (cpm/mg)*
Normal liver	(1)	Hydroxyurea (25 mM) [†]	15	1.3×10^3
	(2)	Hydroxyurea (25 mM)	30	1.0×10^3
	(3)	Hydroxyurea (25 mM)	60	1.1×10^3
	(4)	None	15	2.4×10^3
	(5)	None	30	2.7×10^3
	(6)	None	60	4.2×10^3
Regenerating liver [‡]	(7)	Hydroxyurea (25 mM)	15	6.0×10^3
	(8)	Hydroxyurea (25 mM)	30	5.9×10^3
	(9)	None	15	12.8×10^3
	(10)	None	30	18.4×10^3

*Incubations with [³H-methyl]-thymidine were carried out as described in the text.

[†]All samples were preincubated with hydroxyurea for 30 min. at 37° before addition of [³H-methyl]-thymidine and reincubation for the stated times.

[‡]Liver was removed from the animal 23 hours after 70% partial hepatectomy.⁴

The reason for the increase in the amount of label bound in the hydroxyurea controls of the regenerating liver samples (experiments 7 and 8) compared with that in the normal liver (experiments 1 and 2) is obscure. It cannot be due to DNA synthesis, since there is no change in the value from 15 to 30 min. incubation time, nor is it due to DNA synthesized prior to inhibition, since all cells were preincubated with hydroxyurea before addition of tritiated thymidine. It may reflect a larger population of the proteins capable of binding thymidine in the regenerating liver.

An assay ("phenol" method) avoiding isolation of most of the nonspecifically bound thymidine.

The DNA extraction procedure developed here is based on a previously published method⁹ that was designed to isolate relatively large amounts of "native" DNA. Since our requirements were less stringent with regard to denaturation and minor loss of DNA, we were able to adapt the method to small quantities of DNA (50-400 μ g), and to speed up the isolation to make it more suitable as an assay procedure. The method finally adopted is as follows:

Following the two washes of the cells with EDTA (see above), the cell pellet was treated with 2 ml ice-cold 1 per cent sodium-8-anilino-1-naphthalene sulfonate in 0.2 M sodium acetate, pH 7, and 1 ml 88 per cent liquid phenol. The mixture was shaken for 15 to 30 sec. on a vortex mixer, or until emulsified. The emulsion was then centrifuged at 20,000 rpm at 2° for 5 min. The clear, slightly yellow supernatant solution was pipetted off the phenol layer and added to 3.5 ml of room-temperature absolute alcohol. The mixture was shaken by hand and left at room temperature for 10 min.

The strands of DNA that appear after shaking of the mixture were collected by centrifugation on a bench centrifuge and washed once with 1 ml ice-cold 10 per cent TCA and once with 1 ml room-temperature absolute alcohol. The DNA was then dissolved in 1 ml boiling water and cooled. Aliquots of this solution were used for radioactive counting and colorimetric determination of DNA.

Table 5 shows a comparison of both the "acid precipitation" and phenol assay methods. The results clearly illustrate that both give comparable final specific activities for DNA (when controls have been subtracted), but that the phenol method virtually eliminates the extraction of non-DNA associated tritiated thymidine. The data obtained by the phenol method are likely to be more reliable than the acid method, since the latter relies on small differences between large numbers.

DISCUSSION

It was reported previously¹⁰ that DNA isolated from mouse liver was often contaminated with what appeared to be a glycogen-like material that was capable of binding tritiated thymidine. Our results, however, indicate that, although a small percentage of nonspecifically bound tritiated thymidine in liver cells may be due to the presence of glycogen, most is due to unknown proteins (peptides) in the cells. Indeed, it is claimed that glycogen is extracted from tissues by ice-cold TCA (or perchloric acid),¹¹ and therefore the early acid washes in the "acid precipitation" method should remove glycogen. Furthermore, our other observations on the extent of nonspecifically bound thymidine in regenerating liver and starved rat liver (both known to contain less glycogen than normal liver) showed amounts of protein bound thymidine that were almost comparable to that in normal liver. Glycogen in this case is not the main cause of the nonspecific thymidine binding.

Without further research it is not possible to say which proteins are capable of binding large

Table 5

COMPARISON OF TCA PRECIPITATION AND PHENOL EXTRACTION METHODS*

Sample	Treatment	Total cpm	Specific activity of DNA (cpm/mg)	Δ (Specific activity) [†]
Phenol Method				
No cells	None	40		
Regenerating liver cells	Hydroxyurea (25 mM)	86	1.5×10^3 [†]	
Regenerating liver cells	None	662	10.2×10^3	8.7×10^3
TCA Method				
No cells	None	46		
Regenerating liver cells	Hydroxyurea (25 mM)	361	12.3×10^3 [†]	
Regenerating liver cells	None	958	20.4×10^3	8.1×10^3

*Methods for incubation and assay are described in the text.

[†]"Specific activity" for the hydroxyurea samples does not really refer to DNA, and more properly should be a "protein specific activity"; however, for the purposes of the experiment we have assumed it to be a DNA specific activity.

[‡] Δ (specific activity) is the specific activity of DNA after the contribution due to nonspecific binding (i.e., specific activity of hydroxyurea controls) has been subtracted.

amounts of thymidine in this fashion. It is possible to conjecture, however, that membrane bound proteins do not play a role, since many of these would have been absent from the cell extracts used in the experiments of Tables 2 and 3, although considerable amounts of thymidine were still bound. In addition, since cell extract seemed to be capable of binding more thymidine than an equivalent amount of cytoplasm in the whole cell (Table 3), it is probable that the proteins involved are present in the cytoplasm, but are not able to contact the pool of thymidine within the cell as readily as when they are released by cell rupture into a thymidine-containing medium.

At this point, it might be well to note that errors due to nonspecific binding of thymidine in hepatocytes may account for the discrepancies in specific radioactivities of DNA reported in many studies with liver cell suspensions and also with whole liver.^{12,13} For example, Fisher et al.¹² report large variations in the specific activities of DNA isolated by the acid precipitation method. These could be due to variations among animals, which are hard to control, but might also be due to variations in the extraction procedure or to the lack of suitable controls. In fact, many reports on the use of tritiated thymidine for studies of DNA synthesis do not include details of any controls.¹³ As can be seen from the work reported here, such an omission could be serious if reliable measures of DNA synthesis are sought.

We therefore recommend a combination of both the use of hydroxyurea as an internal control system and adoption of the "phenol" method for DNA extraction in experiments that employ tritiated (or ¹⁴C-labeled) thymidine for measurement of DNA synthesis, especially with hepatocytes. The methods give highly reproducible results and accurate estimates of "real" DNA synthesis.

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STUDIES ON POLYPHENYLALANINE SYNTHESIS WITH A CELL-FREE SYSTEM OF RAT LYMPHOSARCOMA *

By

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The 80 S ribosomes of eukaryotic organisms have been dissociated into subunits active in polypeptide synthesis only relatively recently.^{1,2} Dissociation has been achieved by centrifuging the ribosomes through a sucrose gradient containing a high concentration of salt. Unlike the bacterial system, where Phe-tRNA binding to the small subunit occurs readily in the absence of supernatant enzymes,³⁻⁵ Phe-tRNA binding to the 40 S ribosomal subunit of rat liver has been shown to be dependent on the addition of supernatant factor T1.² More recently, evidence has been presented for the presence of a factor in rat liver supernatant different from T1 which is required along with GTP for maximal binding of Phe-tRNA to the 40 S subunit of rat skeletal muscle.⁶

In the present study, we prepared 40 S and 60 S subunits from rat lymphosarcoma ribosomes isolated by ethanol and Mg^{2+} precipitation and examined polyphenylalanine synthesis, Phe-tRNA binding, and GTP binding and hydrolysis carried out by these subunits. The results are reported here. A preliminary report on part of this work has been presented.⁷

MATERIALS AND METHODS

Preparation of ribosomes. Female albino rats of the Sprague-Dawley strain weighing 75-100 g were used as hosts for Murphy-Sturm lymphosarcoma. The tumor was transplanted by implanting two or three small pieces of tumor tissue (about 2-3 mm thick and 1 cm long) under the skin in the region of the hip. After 7-10 days, the rats were sacrificed, and the tumors were removed and immersed in cold Buffer I containing 10 mM imidazole-HCl, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 250 mM sucrose. All further procedures were carried out at 0-4° unless specified otherwise.

The tumors were minced and homogenized with two volumes of Buffer I. Homogenization was carried out first with a loosely fitting, stainless steel pestle with a flat, serrated tip and then with a more tightly fitting teflon pestle, using 3 or 4 up-and-down strokes. After the extract was centrifuged for 15 min. at 30,000 x g, an amount of 2 M $MgCl_2$ sufficient to obtain a final concentration of 40 mM was added to the clarified extract, which will be referred to as S-30. Then 0.15 volume of -20° ethanol was added while stirring, and the precipitated ribosomes were collected 5 min. later by centrifugation at 25,000 x g for 5 min. The pellets were rinsed quickly and resuspended in Buffer II containing 10 mM imidazole-HCl, pH 7.4, 40 mM KCl, 1 mM $MgCl_2$, and 2 mM 2-mercaptoethanol in a volume equal to one-half that of the S-30. The KCl concentration of the suspension was then increased to 0.5 M by the addition of a 4 M solution of KCl. Twenty minutes later, the $MgCl_2$ concentration of the ribosomal solution was raised to 40 mM, and

* This report is taken from a paper that appeared in *Biochim. Biophys. Acta*, 262:381, 1972.

0.25 volume of ethanol was added. After 5 min., the precipitate was centrifuged at $25,000 \times g$ for 5 min. The ribosome pellet was then rinsed and resuspended in Buffer II by homogenization in a volume equal to one-half that of the original S-30 and clarified after 15 min. by centrifugation at $30,000 \times g$ for 10 min. A final precipitation was carried out by increasing the $MgCl_2$ concentration of the ribosome solution to 40 mM and collecting the ribosomes 15 min. later by centrifugation at $25,000 \times g$. The centrifuged ribosomes were resuspended in Buffer II, clarified by centrifugation, and stored at 0° . For deoxycholate treatment, the ribosomes were resuspended in a solution containing 10 mM imidazole-HCl, pH 7.4, 500 mM KCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.25 per cent sodium deoxycholate just prior to the final precipitation with Mg^{2+} , and precipitated with 8 per cent polyethylene glycol (average mol. wt. 6000) after 15 min. of mixing.

Ribosome concentration was determined from absorbance measurements at 260 nm, assuming 12 units to be equivalent to 1 mg of ribosomes. About 3 mg of ribosomes were obtained per gram wet weight of tissue.

Preparation of subunits. To prepare subunits, 1 ml of a 4-5 mg/ml solution of ribosomes was layered on a 34 ml linear sucrose gradient containing 5-20 per cent sucrose, 10 mM imidazole-HCl, pH 7.4, 500 mM KCl, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol and centrifuged at 10° for 12 hours at 20,000 rev./min. in an International SB110 rotor. Fractions of 1 and 15 drops were collected and the absorbance at 260 nm of the 1-drop fractions was measured after dilution with 1 ml of water. Peak fractions were pooled separately, and the 40 S and 60 S subunits were precipitated with 30 per cent and 25 per cent ethanol, respectively, after the $MgCl_2$ level had been raised to 40 mM. The subunits were collected after 7 min. by centrifugation at $25,000 \times g$ for 8 min.; they were resuspended in Buffer II, and stored at 0° . The subunits prepared in this way and stored at 0° were active for at least 2 weeks.

Preparation of supernatant. Lymphosarcoma or rat liver S-30 extract was centrifuged for 2.5 hours at $321,000 \times g$ in an International A 321 rotor, and the upper two-thirds of the supernatant were passed through a Sephadex G-25 column equilibrated with Buffer II. Peak fractions were combined and stored in small aliquots at -60° .

Preparation of ethanol fraction of supernatant. An additional 0.15 volume of -20° ethanol was added to the supernatant obtained following the precipitation of the ribosomes from tumor S-30 with 0.15 volume of ethanol in the presence of 40 mM $MgCl_2$ (see Preparation of ribosomes). The precipitate was collected after 5 min. by centrifugation at $25,000 \times g$ for 5 min. and resuspended in Buffer II in a volume equal to one-half that of the starting S-30. A second fractionation with ethanol was then carried out, and the material precipitating between 0.10 and 0.25 volumes of ethanol was collected. The ethanol precipitate was dissolved in Buffer II with 5 per cent glycerol in a volume equal to one-quarter of the starting S-30, and stored at -60° .

Preparation of T1 and T2. All phosphate buffers used contained 2 mM 2-mercaptoethanol and 5 per cent glycerol. About 5 mg of calcium phosphate gel (dry weight) per mg protein were added to the ethanol fraction of the supernatant with mixing. After 15 min. of stirring, the mixture was centrifuged for 5 min. at $5000 \times g$, and the pelleted gel was washed twice by homogenization in 0.05 M phosphate buffer, pH 7.0, and centrifugation. T2 was then eluted from the gel by 5 extractions with 0.125 M phosphate buffer, each extraction involving homogenization of the gel in a volume of phosphate buffer equal to one-half that of the ethanol fraction, and centrifugation after stirring for 10 min. The active fractions (usually the first three) were pooled, and re-

sidual T1 activity was removed by treating the pooled extract with a fresh batch of calcium phosphate gel, excess water being removed from the gel by centrifugation before adding the extract. T1 was eluted in a similar manner to T2 by three extractions with 0.25 M phosphate. The separated factors were dialyzed against Buffer II with 5 per cent glycerol and stored at -60° .

Preparation of [^{14}C]Phe-tRNA. [^{14}C]Phe-tRNA of specific activity $375\ \mu\text{Ci}/\mu\text{mole}$ and non-radioactive Phe-tRNA were prepared from *Escherichia coli* tRNA, as described previously.⁸ For experiments demonstrating the dependence of Phe-tRNA binding to ribosomes on GTP or 5'-guanyl methylene diphosphonate (GDPCP), the Phe-tRNA preparation was passed through a Sephadex G-25 column.

Assay for Phe-tRNA binding. The reaction mixture, unless otherwise specified, contained the following in a final volume of 0.25 ml: 50 mM imidazole-HCl, pH 7.4, 40 mM KCl, 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, and 0.2 mM GTP. [^{14}C]Phe-tRNA, poly U, supernatant enzymes and ribosomes were added as indicated. The reaction was stopped with 0.01 ml of formaldehyde (37 per cent solution) after incubation at 37° . This was followed immediately by the addition of 2.5 ml of ice-cold buffer containing 10 mM imidazole-HCl, pH 7.4, 80 mM KCl, and 8 mM MgCl_2 . The ribosomal complex was then adsorbed onto Millipore filter, washed, and measured for radioactivity in a Nuclear-Chicago gas flow counter unless stated otherwise.

Materials. Rat liver tRNA was purchased from General Biochemicals, and GDPCP and poly U were from Miles Laboratories. Fusidic acid was a generous gift from Dr. Josef Fried. Heparin, purified under grant HE-11083 of the National Heart Institute (NIH), was kindly provided by Dr. L. Roden.

RESULTS

Ribosomes were prepared from the lymphosarcoma by ethanol and Mg^{2+} precipitation and dissociated into 40 S and 60 S subunits by centrifuging through a sucrose gradient containing 0.5 M KCl. The results of such an experiment are shown in Figure 1. Fractions collected from the top of the gradient were low in absorbance, indicating that the 80 S ribosomes prepared without ultracentrifugation were essentially free of contaminating RNA and proteins. Vigorous synthesis of polyphenylalanine was observed only when the fractions containing 40 S and 60 S subunits were combined. Although the procedure yielded 40 S subunits relatively free of 60 S ribosomal subunits, the purity of the fractions containing the 60 S subunits was more variable. Contamination of the 60 S subunit fractions with 40 S subunits by as much as 10-15 per cent has been observed. When recombined, the dissociated ribosomes were usually more active for polyphenylalanine synthesis than were the parent 80 S ribosomes.

The 40 S ribosomal subunits were active in binding Phe-tRNA in the presence of poly U, GTP, and rat liver or tumor supernatant (Table 1). The omission of GTP or of supernatant markedly reduced the amount of Phe-tRNA bound. GDPCP substituted effectively for GTP, indicating that cleavage of GTP was not obligatory in the binding. Fusidic acid at 10^{-3} M, a concentration at which polyphenylalanine synthesis is inhibited by more than 90 per cent,⁹ had no effect on the binding of Phe-tRNA to the 40 S subunit.

The binding of Phe-tRNA to the 40 S ribosomal subunit was a fairly rapid reaction, as seen in Figure 2, being essentially complete in about 4 min. The increase in Phe-tRNA binding with the addition of more 40 S subunits after 5 min. of incubation indicated that the ribosomes were the limiting component in the reaction.

Figure 1. Isolation of ribosomal subunits by sucrose density gradient centrifugation. The assay mixture for polyphenylalanine synthesis contained in 0.25 ml, 50 mM imidazole-HCl, pH 7.4, 80 mM KCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM ATP, 1 mM GTP, 2 mM phosphoenolpyruvate, 2 μ g of pyruvate kinase, 50 μ g of rat liver tRNA, 40 μ g of poly U, 0.015 ml of supernatant, 80 μ g of [^{14}C]Phe-tRNA of specific activity 375 μ Ci/ μ mole, 0.02 ml of gradient fraction and, where indicated, 0.02 ml of pooled Fractions 32 and 34. Incubation was for 10 min. at 37°. Radioactivity in hot trichloroacetic acid precipitable material was measured in a Nuclear-Chicago gas flow counter and is represented by broken-line curves.

Table 1
DEPENDENCE OF Phe-tRNA BINDING TO 40 S SUBUNIT ON
SUPERNATANT AND GTP

Condition	[^{14}C]Phe-tRNA bound (counts/min.)		
	Expt. 1	Expt. 2	Expt. 3
Complete	388	282	362
Minus poly U	3	5	-
Minus GTP	92	77	110
Minus supernatant	17	13	-
Plus GDPCP, minus GTP	-	-	409
Plus fusidic acid	-	-	380

The assay was carried out as described in the text. Ten μ g of 40 S subunits, 80 μ g of [^{14}C]Phe-tRNA of specific activity 375 μ Ci/ μ mole and, where indicated, 0.4 mM GDPCP, 0.4 mM GTP, 0.02 ml of supernatant, 25 μ g of poly U, and 0.15 mM fusidic acid were added. Incubation was for 5 min.

Figure 2. Time course of Phe-tRNA binding to 40 S subunit. The reaction mixture contained 0.02 ml supernatant, 8 mM MgCl₂, 80 mM NH₄Cl, 10 µg of 40 S subunits, 10 µg of poly U, and 80 µg of [¹⁴C]Phe-tRNA. Other components and conditions of the assay were as described in the text.

The requirement for poly U in the binding of the Phe-tRNA to the 40 S subunit is shown in Figure 3. Phe-tRNA binding was about two-thirds of the maximal rate with 1 µg of poly U.

Figure 3. Poly U requirement for Phe-tRNA binding to 40 S subunit. The assay conditions were similar to those described in Table 1 except that the concentration of poly U was varied, and GTP and supernatant were included in all tubes.

Unlike the binding of Phe-tRNA to the small subunit of bacterial ribosomes, which occurs readily without supernatant or GTP,³⁻⁵ the binding of the Phe-tRNA to the 40 S subunit of the lymphosarcoma was dependent on supernatant over a wide range of Mg^{2+} concentrations (Figure 4). Two optima of Mg^{2+} concentration were observed. The first and lower optimum occurred at about 8 mM Mg^{2+} and coincided with the range that was optimal for polyphenylalanine synthesis. Although the second optimum, observed at 15-18 mM of Mg^{2+} , resulted in greater binding of Phe-tRNA, polyphenylalanine synthesis in this range of Mg^{2+} was much less than at 8 mM.

Figure 4. Effect of supernatant on Phe-tRNA binding to 40 S subunit at varying $MgCl_2$ concentrations. Assay conditions were as described in Figure 3 except for a fixed poly U concentration of 25 μ g and varying amounts of $MgCl_2$.

Phe-tRNA binding studies carried out with partially purified supernatant factors indicated that the T1 was involved in the binding of the aminoacyl-tRNA to the 40 S subunit (Table 2). As with the crude supernatant, maximal binding occurred only in the presence of the supernatant protein, T1 in this case, and GTP. T2, which was separated from T1 by fractionation on calcium phosphate gel, did not stimulate the reaction. Fusidic acid, at a concentration that inhibited polyphenylalanine synthesis by 90 per cent or more, again had no effect on the binding reaction.

Although the GTP analogue, GDPCP, was as effective as GTP in the binding of Phe-tRNA to the 40 S subunit, it was about half as active as GTP when the 60 S subunit was present together with the 40 S subunit (Table 3). The level of binding attained with GDPCP in the latter case was approximately equal to that reached with the 40 S subunit only. The results indicate that additional binding sites were made available with GTP, but not with GDPCP, conceivably because Phe-tRNA was translocated by T2 still contaminating the ribosomes. GDPCP was much less effective in Phe-tRNA binding to undissociated 80 S ribosomes than to the combination of dissociated 40 S and 60 S subunits. The reason for this is not clear. The observation on Phe-tRNA binding to 80 S ribosomes with GDPCP is more or less in accord with that made by Lin et al,¹⁰ who

Table 2
DEPENDENCE OF Phe-tRNA BINDING TO 40 S SUBUNIT
ON T1 FRACTION

Condition	[¹⁴ C]Phe-tRNA bound (counts/min.)
Complete	370
Minus poly U	31
Minus T1	22
Minus GTP	69
Minus T1, plus T2	37
Plus 0.15 mM fusidic acid	374

Assay conditions for the complete system were as described in Figure 2 except for the substitution of 0.02 ml of T1 fraction for supernatant, and a fixed time of incubation of 5 min. Where indicated, 0.02 ml of a T2 fraction was added.

Table 3
EFFECT OF GDPCP ON Phe-tRNA BINDING

Condition	[¹⁴ C]Phe-tRNA bound (counts/min.)		
	40 S subunits	40 S plus 60 S subunits	80 S ribosomes
Complete	634	1545	1787
Minus GTP	160	197	145
Minus GTP, plus GDPCP	639	738	340
Minus poly U	-	131	156

The assay conditions were similar to those described in Table 2 except for the presence of 0.3 mM fusidic acid and the addition, where indicated, of 20 μ g of 40 S subunits, 39 μ g of 60 S subunits, 50 μ g of 80 S ribosomes, 0.2 mM GTP, and 0.2 mM GDPCP.

reported that GDPCP did not fully substitute for GTP in Phe-tRNA binding to 80 S ribosomes of rabbit reticulocyte cells.

In earlier studies carried out with heparin as an inhibitor of protein synthesis, fairly high concentrations were used.¹¹ In the present study, the effect of much lower concentrations of heparin on polypeptide synthesis was examined (Figure 5). Binding of Phe-tRNA to the 40 S ribosomal subunit was more sensitive to inhibition by heparin than binding to the 80 S ribosomes. At 10^{-6} M heparin, binding of Phe-tRNA to the 40 S subunit was inhibited by about 90 per cent in the experiment represented in Figure 5. In other experiments, total inhibition of binding was observed with 10^{-6} M heparin. Binding of Phe-tRNA to the 80 S ribosomes, on the other hand, was inhibited by only 50 per cent at 10^{-6} M heparin, and a 10-fold increase in the concentration of heparin did not increase the inhibition further. Inhibition of polyphenylalanine synthesis by heparin paralleled the inhibition of Phe-tRNA binding to the 80 S ribosomes rather than to the 40 S subunits. About half the synthetic activity of the 80 S ribosomes appeared to be more sensitive to heparin.

Figure 5. Effect of heparin on polyphenylalanine synthesis and Phe-tRNA binding. Polyphenylalanine synthesis was assayed in a reaction mixture containing 50 mM imidazole-HCl, pH 7.4, 8 mM MgCl₂, 80 mM NH₄Cl, 25 μ g of poly U, 10 mM 2-mercaptoethanol, 1 mM GTP, 0.015 ml of ethanol fraction of supernatant, 47 μ g of deoxycholate-washed ribosomes, and 54 μ g of [¹⁴C]Phe-tRNA. Incubation was for 8 min. at 37°. The assay conditions for Phe-tRNA binding were as described in Table 2, except that incubation was for 8 min. and heparin was added. Where indicated, 47 μ g of deoxycholate-treated ribosomes or 19 μ g of 40 S ribosomes were added.

The effective substitution of GTPCP for GTP in Phe-tRNA binding to the 40 S subunit suggested that GTP hydrolysis was not obligatory in the reaction. To determine whether this was actually the case, we carried out the binding experiments with [γ -³²P]GTP. As seen from Table 4, [γ -³²P]GTP binding to ribosomes, as measured by retention of radioactivity on Millipore filter, occurred along with Phe-tRNA binding. Maximal radioactivity was retained on the filter when all the components of the binding reaction were included in the system. Approximately equimolar amounts of GTP and Phe-tRNA were bound. Omission of any one of the components resulted in a decrease of both GTP and Phe-tRNA binding. The high baseline observed in GTP binding in the absence of 40 S subunits appears to be due to the interaction of T1 or some other component in the T1 preparation with either GTP or a radioactive contaminant of GTP. Heparin and antibiotics that inhibited binding of Phe-tRNA also inhibited binding of GTP to varying extents. The inclusion of the 60 S subunit in the reaction mixture substantially diminished the amount of GTP bound, presumably by promoting hydrolysis of GTP.

To demonstrate that GTP and Phe-tRNA were actually bound to the 40 S subunits, the incubated samples were centrifuged through sucrose gradients (Figure 6). In spite of the substantial aggregation of the 40 S subunits brought about by the addition of poly U, it is clear from the data that GTP and Phe-tRNA were bound to the 40 S subunits (left and right panels, respectively). Stimulation of Phe-tRNA binding by GTP was small in this experiment, probably because of the relatively low concentration of GTP added to the system.

Table 4
BINDING OF GTP AND Phe-tRNA TO 40 S SUBUNIT

Condition	$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ bound (counts/min.)	$[^{14}\text{C}]\text{Phe-tRNA}$ bound (counts/min.)
Complete	1952	2278
Minus 40 S subunit	430	113
Minus T1	99	280
Minus poly U	479	120
Minus Phe-tRNA	503	-
Minus Phe-tRNA, plus uncharged tRNA	587	-
Plus 0.31 mM chlortetracycline	783	491
Plus 3 mM streptomycin sulfate	443	725
Plus 10 μM heparin	605	93
Plus 60 S subunit	235	-

The reaction mixture for Phe-tRNA binding contained 60 nM GTP and 20 μg of 40 S subunits. Other components of the reaction, as well as the conditions of the assay, were as described in Table 2. The GTP binding assay was identical to that for Phe-tRNA binding except that 60 nM of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ of specific activity 356 $\mu\text{Ci}/\mu\text{mole}$ and 60 μg of non-radioactive Phe-tRNA were substituted for unlabeled GTP and $[^{14}\text{C}]\text{Phe-tRNA}$. Where indicated, 60 μg of uncharged *E. coli* tRNA and 39 μg of 60 S subunits were added. Radioactivity was measured by placing the dried Millipore filters in toluene scintillation fluid and counting in a Beckman scintillation counter.

The results presented in Table 5 show that binding of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ to 40 S subunits was diminished by 60 S subunits because of hydrolysis of the GTP. Both ribosomal subunits and Phe-tRNA were required for the T1-catalyzed GTPase activity. Poly U, although required for Phe-tRNA binding (see Table 3), was not essential for the reaction, a slight inhibition by poly U being observed in this instance. The hydrolysis of GTP, in other words, was not dependent on Phe-tRNA binding. Similar observations have been made with rabbit reticulocyte and *E. coli* ribosomes, using highly purified aminoacyl-tRNA binding factors.^{10,13} This would explain the inability of chlortetracycline to inhibit hydrolysis, and the relatively mild inhibition by streptomycin even though both antibiotics are strong inhibitors of aminoacyl-tRNA binding.^{14,15} Heparin inhibited both binding of Phe-tRNA and the GTPase activity. Fusidic acid, which inhibits T2-catalyzed GTPase, did not affect the T1-dependent hydrolysis of GTP.

Finally, since factors other than T1 have been reported to stimulate Phe-tRNA binding to 40 S and 80 S ribosomes,^{6,16,17} a T1 preparation similar to those used throughout this study was chromatographed on Sephadex G-200 gel. Assays of the fractions obtained showed that the fractions with T1 activity, identified by stimulation of polyphenylalanine synthesis in the presence of T2, were rather weakly active in inducing Phe-tRNA binding to the 40 S subunit unless they were supplemented with material eluting later from the column (Figure 7). The new component was not effective by itself in stimulating Phe-tRNA binding; it did not appear to be required for polyphenylalanine synthesis with 80 S ribosomes. The new factor was not present in the T2 preparation used in the assay for polyphenylalanine synthesis.

Figure 6. Demonstration of GTP and Phe-tRNA binding to the 40 S subunit. Curves in the left panel represent $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$ binding to 40 S subunit and those in the right, $[\text{C}^{14}]\text{Phe-tRNA}$ binding. The reaction mixture for GTP binding contained 160 μg of 40 S subunits, 0.23 μM of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ of specific activity 426 $\mu\text{Ci}/\mu\text{mole}$, and other components as described in Table 4. Where indicated, 50 μg of poly U and 120 μg of nonradioactive Phe-tRNA were added. Phe-tRNA binding was carried out under similar conditions except that 68 μg of $[\text{C}^{14}]\text{Phe-tRNA}$ and nonradioactive GTP were added. After a 10 min. incubation, the reaction mixture was layered on a 4 ml, 5-20% sucrose gradient containing 10 mM imidazole-HCl, pH 7.4, 100 mM NH_4Cl , 20 mM MgCl_2 , and 10 mM 2-mercaptoethanol, and centrifuged for 45 min. at 56000 rev./min. in a Spinco SW 56 rotor. Alternate 2-drop fractions were collected for absorbance measurements and 3-drop fractions for radioactivity measurements. Radioactivity was measured in a Beckman scintillation counter using Bray's¹² solution. Absorbance is represented by solid-line curves, and radioactivity by broken-line curves. Direction of sedimentation is from right to left of the figure.

Figure 7. Separation of 40 S subunit binding activity of the T1 preparation into two fractions. Two ml of a 0-65% $(\text{NH}_4)_2\text{SO}_4$ fraction of the calcium phosphate gel T1 was applied to a Sephadex G-200 column (2.5 cm x 35 cm) equilibrated with the elution buffer containing 10 mM imidazole-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, and 10 mM 2-mercaptoethanol. 1.8 ml fractions were collected at a flow rate of 18 ml/hour. Assay conditions were as described in Table 2 except for the substitution of column fractions for T1. Where indicated, 0.05 ml of Fraction 57 was added to the reaction mixture with 0.01 ml of the early fractions (27-40) and 0.01 ml of Fraction 30 was added with 0.05 ml of the late fractions (49-70). The broken-line curves with open squares represent the amount of radioactivity bound with aliquots of the indicated fractions alone, and the curves with open circles, the amount of radioactivity bound when the fractions were supplemented with either Fraction 57 or 30.

Table 5
REQUIREMENTS FOR GTP HYDROLYSIS

Condition	$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ hydrolyzed (%)
Complete	65.7
Minus Phe-tRNA	1.2
Minus 40 S subunit	11.8
Minus 60 S subunit	3.6
Plus poly U	59.9
Plus chlortetracycline	70.6
Plus streptomycin sulfate	42.1
Plus heparin	17.1
Plus fusidic acid	67.2

The reaction mixture was essentially as described in Table 2 except for the omission of $[^{14}\text{C}]\text{Phe-tRNA}$ and the presence of $0.32\text{ }\mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ of specific activity $72\text{ }\mu\text{Ci}/\mu\text{mole}$. Where indicated, $60\text{ }\mu\text{g}$ of Phe-tRNA, $20\text{ }\mu\text{g}$ of 40 S subunits, $39\text{ }\mu\text{g}$ of 60 S subunits, 0.02 ml of T1, $15\text{ }\mu\text{g}$ of poly U, $50\text{ }\mu\text{g}$ of *E. coli* tRNA, 0.3 mM fusidic acid, 0.31 mM chlortetracycline, 3 mM streptomycin sulfate, and $10\text{ }\mu\text{M}$ heparin were added. After 5 min. of incubation at 37° , the reaction was stopped with 2 ml of cold 5% trichloroacetic acid containing 2% charcoal (w/v). The samples were then kept in ice with occasional mixing for 10 min., after which the charcoal was collected on a Millipore filter and washed with cold water. Radioactivity adsorbed onto the charcoal, representing unhydrolyzed GTP, was measured in a Nuclear-Chicago gas flow counter.

DISCUSSION

The procedure which we have developed for isolating ribosomes from rat lymphosarcoma by ethanol and Mg^{2+} precipitation is simple and rapid. The 80 S ribosomes and the subunits derived from these ribosomes are very active in polypeptide synthesis.

The 40 S ribosomal subunits of the tumor differ from those of the 30 S subunits of *E. coli* in that binding of Phe-tRNA to the tumor subunits with poly U is strongly dependent on GTP and the supernatant factors. A requirement for GTP and T factor in the binding of Phe-tRNA to the bacterial ribosomes has been demonstrated thus far only with undissociated 70 S ribosomes.^{18,19} Recently, Leader et al.⁶ have presented some evidence for the existence of a factor in rat liver supernatant different from T1 which stimulated Phe-tRNA binding to the 40 S ribosomal subunit of rat skeletal muscle in the presence of GTP. Although T1 induced some Phe-tRNA binding, little or no stimulation by GTP was seen with T1. Moldave and Gasior,¹⁶ in a preliminary report, have mentioned the separation of a T1 preparation partially purified from rat liver supernatant into three components with different binding activities. One of these components, different from T1, catalyzed Phe-tRNA binding to the 40 S ribosomal subunit. Our observations differ somewhat from these reports: we find that both T1 and the second supernatant factor are required along with GTP for Phe-tRNA binding to the 40 S subunit. T1 alone, or the second supernatant factor alone, is weakly active.

A GTPase activity dependent on highly purified T1 and 80 S reticulocyte ribosomes was demonstrated by Lin et al.¹⁰ Phe-tRNA was reported to be required for the reaction, but not poly U. The present study shows that the lymphosarcoma ribosomes display a similar, uncoupled GTPase activity which is dependent on T1. In addition, we have shown that the T1-dependent

GTPase activity which is coupled to Phe-tRNA binding requires both ribosomal subunits. In the absence of the 60 S subunit, GTP is bound without hydrolysis to the 40 S subunit along with Phe-tRNA.

The results of the studies with antibiotics and heparin on the GTPase activity are in accord with the view that there is a close relationship between the T1-catalyzed GTPase activity and the ribosomal binding of aminoacyl-tRNA. Fusidic acid, which inhibits translocation and the T2-catalyzed GTPase activity without affecting the T1-dependent binding of Phe-tRNA, had no effect on the T1-dependent GTPase activity. Heparin, which we have found to be a fairly effective inhibitor of Phe-tRNA binding to the 40 S subunit, inhibited the T1-catalyzed GTPase activity. Although the inhibition of Phe-tRNA binding by chlortetracycline without appreciable inhibition of the GTPase activity appears discordant with the above results, the observation may simply be a consequence of the uncoupling of GTP hydrolysis from aminoacyl-tRNA binding.

The need for GTP in the translocation step of polypeptide synthesis seems obvious enough, since energy must be provided for shifting of the peptidyl-tRNA and mRNA on the ribosome. The need for GTP in the binding of aminoacyl-tRNA, on the other hand, appears less obvious. The effectiveness of GTP in the binding reaction indicates that binding of aminoacyl-tRNA can occur without utilization of the hydrolytic energy of GTP. The question of interest is whether cleavage of GTP is required to drive some reaction after aminoacyl-tRNA is bound, or whether it is simply a means for releasing and regenerating free T1 once aminoacyl-tRNA is properly bound.

Because of the strong dependence of Phe-tRNA binding to the 40 S ribosomal subunit on supernatant factors and GTP, the mammalian system may turn out to be useful for studying the role of GTP in aminoacyl-tRNA binding. Further work on this problem, as well as an examination of the functional role of the new supernatant factor, is being carried out in our laboratory.

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SYNERGY OF ANTIHOST ANTISERUM WITH DONOR SPLEEN CELLS IN THE GRAFT-VERSUS-HOST REACTION*

By

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The graft-versus-host (GVH) reaction is an immune response of grafted lymphoid cells against histocompatibility antigens of their host. Proliferation of lymphoid cells and hypertrophy of lymphatic organs have been described as early manifestations of the GVH reaction.^{1,2}

Uhr and Möller have discussed the role of endogenously produced antibody in the regulation of the immune response.³ The use of passively administered antibody to suppress rejection has been successful for tumor grafts,⁴ and passive antibody given together with antigen has suppressed rejection of kidney grafts⁵ and prolonged skin graft survival.⁶ Voisin and Kinsky suggested that the GVH response might be suppressed by antihost antibody in a fashion analogous to enhancement of tumor by antitumor antibody.⁷ There have been several reports of mitigation of the GVH reaction by antihost antiserum.⁷⁻¹⁰ In these studies, death of the host usually was made the end point.

The experiments reported here were originally undertaken to extend the observations of GVH suppression by antihost antibody. The spleen weight model of the GVH response, as described by Simonsen,¹ was chosen as a rapid quantitative assay system. The observed synergy of the antihost antiserum with the GVH response was unexpected. Such a synergistic effect has been reported by Batchelor and Howard.⁸ They compared this result to the synergy of antitumor antibody in rejection of a spleen cell tumor graft,¹¹ much as Voisin and Kinsky had noted a similarity between tumor enhancement and GVH suppression. Synergy of antibodies with the active immune response is not easily distinguished from direct effects of antibodies on target tissues. It is probably for this reason that synergistic effects have not been more frequently reported.

MATERIALS AND METHODS

Animals

Adult A/He and CBA mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. F₁ hybrids were produced by mating A/He female with CBA male mice.

Cell Suspensions

Adult mouse spleens moistened with Hanks' balanced salt solution without NaHCO₃ (HBSS; Grand Island Biological Co., Grand Island, New York) were placed on a 0.5 mm mesh stainless steel screen, disrupted with a porcelain pestle, and washed into a plastic Petri dish with HBSS. Nucleated cells were counted in a Coulter counter; a yield of 10⁸ cells per spleen was commonly

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obtained. When used as antigen for alloantiserum preparation, this suspension was given in a dose of one spleen equivalent per mouse.

If the cells were to be injected into hybrid mice to elicit a GVH response, the suspension was centrifuged 3 times at 110 g for 30 sec., and the pellets were discarded. It was then sedimented 3 times at 700 g for 5 min., the supernatant was discarded each time, and the cells were resuspended in fresh HBSS. Centrifugation was done at 4°C. For injection, cells were suspended in either HBSS or undiluted serum. Unsensitized donors were used in all GVH experiments. When recipients were paired, both mice received cells from the same preparation. When adult hybrid spleen cells were required as cell controls, the hybrid cells were prepared in parallel with parental cells and were adjusted to the same final cell concentration by the addition of HBSS.

Alloantibody Preparation

IaCBA antibody. A group of 40 adult A/He female mice was given a series of 7 i.p. injections of 10^8 spleen cells from adult CBA mice; injections were made at 2 to 2-1/2-week intervals. Ten to fifteen days after the fifth and sixth doses, each animal was bled approximately 0.25 ml from the retroorbital venous plexus. The serum (designated as IaCBA) was pooled and stored at -20°C.

The immunized mice were then given three injections of Freund's complete adjuvant (0.5 ml/dose) (Difco Laboratories, Inc., Detroit, Michigan) i.p. at 4 to 8-day intervals to elicit ascitic fluid.⁹ Adjuvant injections were begun 3 days before administration of the seventh and last dose of CBA spleen cells. During 1-1/2 months of ascitic-fluid production, the group yield was 150 ml. The fluid was allowed to clot overnight at 4°C and was then centrifuged at 700 g for 5 min. Next, the supernatant was centrifuged at 123,000 g for 45 min. The upper one-third of the fluid containing white, sparingly miscible material was discarded, and the lower two-thirds of clear yellow fluid (IaCBA ascitic fluid) was stored at -20°C. The yield after centrifugation was approximately 100 ml.

IIaCBA antibody. This second pool of alloimmune ascitic fluid was prepared in a fashion identical to the first, except that (1) a total of eight immunizing doses of CBA spleen cells was given; (2) the first five doses contained 2×10^7 cells, and only the last three contained 10^8 cells; and (3) these mice were not bled during the immunization procedure. Respective yields of 240 and 150 ml before and after ultracentrifugation were obtained.

Hemagglutinin titers were determined by the capillary migration technique of Severson and Thompson.¹² Titers are expressed as the reciprocal of the maximum dilution that agglutinates CBA erythrocytes.

Control serum and ascitic fluid were obtained from unimmunized adult female A/He mice. Absorptions of undiluted IIaCBA ascitic fluid were performed by three sequential incubations with washed spleen cells. Incubations were done at 4°C for 2 hours. One spleen equivalent per ml of ascitic fluid was used for each incubation.

Injection Methods

For the spleen weight model, injections were given i.p. in 3 to 9-day-old mouse litters, according to a protocol described by Michie.¹³ After obvious runts had been discarded, each litter was weighed collectively. Each mouse received cells either as a fixed volume of 0.10 ml or as a dose of 0.05 ml/g of average mouse weight. A/He cell doses were in the range of $4.3\text{--}13.1 \times 10^6$ cells/g; CBA cell doses were $4.8\text{--}6.4 \times 10^6$ cells/g. Undiluted ascitic fluid was given in 0.10 ml

amounts one day before and three or four days after cell injections; alternately, approximately 0.10 ml of antiserum was given mixed with cells.

To elicit the GVH reaction in irradiated adult hybrid mice, we injected cell doses in 0.3 or 0.4 ml volumes of HBSS into the lateral tail vein, in doses of up to 64×10^6 cells per mouse. Ascitic fluid or serum was given in 0.5 ml amounts by i.p. injection 1 day before and 3-5 days after cell injection.

Irradiation

Adult hybrid mice were individually enclosed in 50 ml plastic cylinders placed on a rotating Lucite platform. A GE Maxitron 250 was the source of radiation. Total-body x-rays were delivered at a rate of 60 R/min. at 250 keV and 30 mA, with a filter of 0.25 mm Cu and 1.00 mm Al and a source-to-target distance of 77 cm. Mice were irradiated one day before cell injections.^{14,15}

Spleen Weights

Individual mice were weighed to the nearest 0.1 g, and their spleens were weighed to the nearest mg at 10-14 days after cell injection.

Statistical Methods

In the spleen weight model, two mice were compared. For each comparison, the test mouse received a preparation that might affect spleen weight, and the standard mouse received a corresponding inert preparation. Paired mice were litter mates of the first-generation hybrid between two inbred strains of mice.¹³ Where two or more pairs came from the same litter, members of each pair within the litter were randomly assigned. Both members of a pair were treated in identical fashion, except for the experimental variable in question.

Comparisons were made by calculating the difference (D) between relative spleen weights (RSW) of standard and test animals,¹ as:

$$RSW = \text{spleen weight (mg)} / \text{body weight (g)},$$

and $D = RSW \text{ of test mouse} - RSW \text{ of standard mouse}.$

To combine data from a group of such mouse pairs, we first calculated the value of D for each mouse pair. Next, the mean value, $\bar{D}$, was computed. The Student's *t* test method¹⁶ was used to compute the probability (P) that $\bar{D}$ is not significantly different from zero.

In the adult mortality model, survival times (ST) of test and standard mice were compared. ST was measured in days, with the day of cell injection counted as day zero. Test and standard mice were paired at the beginning of the experiment according to age and sex. Both members of a pair were irradiated and caged together and were given cells from the same preparation.

The quantity ST was treated essentially in the same way as was RSW in the spleen weight model:

$$D' = ST \text{ of test mouse} - ST \text{ of standard mouse}.$$

The Student's *t* test method was used to compute the value of P for the mean $\bar{D}'$.

RESULTS

Spleen Weight Model

The effect of A/He spleen cells on the spleen weight of (A/He x CBA)*F*₁ hybrid infant mice

was demonstrated by giving test mice A/He spleen cells, and by giving standard mice equal doses of hybrid spleen cells. All of these mice received control serum (Table 1, Index No. 1). Mice receiving A/He cells had larger spleens than did hybrid cell recipients ($P < 0.001$). For 14 mouse pairs, the mean difference in RSW was 5.1 mg/g. This finding of splenomegaly attributable to parent strain cells alone is consistent with the lymphoproliferative form of the GVH response.¹

The effect of anti-CBA preparations in the absence of a GVH reaction was tested in littermate pairs receiving hybrid spleen cells. Test mice were given anti-CBA as serum or ascitic fluid (Table 1, Index Nos. 2, 3, and 4). Among six pairs, no significant difference in spleen weight was found between mice given Π aCBA ascitic fluid and those given control ascitic fluid ($P > 0.975$); among four pairs, no difference was found between mice given IaCBA serum and those given control serum ($P > 0.7$). These data indicate that anti-CBA antibody alone did not alter spleen weight. Among nine mouse pairs receiving hybrid spleen cells, no significant difference in spleen weight was found between mice given IaCBA or Π aCBA ascitic fluid and mice given control serum ($P > 0.7$). This indicates that ascitic fluid injections did not alter spleen weight in the absence of a GVH reaction, regardless of antibody content.

The effect of anti-CBA preparations on the spleen weight of mice undergoing a GVH response was examined in littermate pairs given A/He spleen cells. Test mice were given IaCBA serum or ascitic fluid, whereas standard mice received control serum (Table 1, Index Nos. 5 and 6). Fifteen test mice given IaCBA serum with the cells had larger spleens than did standard mice ($P < 0.001$). The mean difference in RSW was 3.01 mg/g. Twelve test mice given IaCBA ascitic fluid separately from the cells had larger spleens than standard mice ($P < 0.02$). The mean difference in RSW was 1.94 mg/g. These results indicate that A/He anti-CBA preparations had a spleen-enlarging effect in (A/He $\times$ CBA) F_1 hybrid infant mice undergoing a GVH reaction. This is in contrast with the failure of anti-CBA preparations to alter spleen weight in hybrid cell recipients.

The effect of absorption with CBA or A/He spleen cells upon the spleen-enlarging quality of anti-CBA ascitic fluid was tested in littermate pairs given A/He spleen cells (Table 1, Index Nos. 7 and 8). Nineteen test mice receiving Π aCBA ascitic fluid absorbed with A/He cells had larger spleens than did standard mice given the identical anti-CBA preparation absorbed with CBA spleen cells ($P < 0.02$). The mean difference in RSW was 1.54 mg/g. Among 21 mouse pairs, however, there was no difference in RSW between mice injected with Π aCBA ascitic fluid absorbed with A/He cells and those given unabsorbed Π aCBA ascitic fluid ($0.3 < P < 0.4$). These results indicate that splenomegaly attributable to anti-CBA ascitic fluid was specifically reduced by absorption with CBA spleen cells.

The effect of anti-CBA ascitic fluid on the spleen weight of littermate pairs given CBA spleen cells was examined (Table 1, Index No. 9). Undiluted IaCBA ascitic fluid was given in two 0.1 ml injections, one day before and four days after cell injection. Seventeen test mice receiving anti-CBA ascitic fluid had larger spleens than did standard mice ($P < 0.01$). The mean difference in RSW was 1.56 mg/g. These results indicate that the spleen-enlarging effect of anti-CBA preparations occurred even when immune cells and antibodies were directed against different antigenic determinants.

Adult Mortality Model

The requirement for parent strain cells in the "GVH death" of adult hybrid mice was examined. Four sets of eight hybrid mice grouped according to age and sex were given 360 R of total-

Table 1

THE EFFECT OF A/He ANTI-CBA ALLOANTISERUM ON THE RELATIVE SPLEEN WEIGHT OF INFANT
(A/He x CBA) F_1 HYBRID MICE GIVEN PARENT STRAIN SPLEEN CELLS

Index no.	No. of mouse pairs	$\bar{D}^*$ (mg/g)	$P^\dagger$	Test mouse antibody preparation			Standard mouse antibody preparation		
				Type	Titer ‡	Cells	Type	Titer ‡	Cells
1	14	5.10	<0.001	Control serum	-	A/He	Control serum	-	Hybrid
2	6	-0.03	>0.975	Π aCBA ascitic fluid	128	Hybrid	Control ascitic fluid	-	Hybrid
3	4	-0.15	>0.7	IaCBA serum	4096	Hybrid	Control serum	-	Eybrid
4	9	-0.14	>0.7	IaCBA or Π aCBA ascitic fluid	256 or 128	Hybrid	Control serum	-	Hybrid
5	15	3.01	<0.001	IaCBA serum	4096	A/He	Control serum	-	A/He
6	12	1.94	<0.02	IaCBA ascitic fluid	256	A/He	Control serum	-	A/He
7	19	1.54	<0.02	Π aCBA ascitic fluid absorbed with A/He cells	128 §	A/He	Π aCBA ascitic fluid absorbed with CBA cells	128 §	A/He
8	21	-0.60	0.3-0.4	Π aCBA ascitic fluid	128	A/He	Π aCBA ascitic fluid absorbed with A/He cells	128 §	A/He
9	17	1.56	<0.01	IaCBA ascitic fluid	256	CBA	Control serum	-	CBA

* $\bar{D}$ is the mean value of individual differences in relative spleen weight of mouse pairs.

$^\dagger P$ is the probability that $\bar{D}$ is not significantly different from zero.

‡ Hemagglutinin titers are expressed as the reciprocal of the maximum dilution that agglutinates CBA erythrocytes.

§ Π aCBA ascitic fluid was titered only before absorption with cells.

body x-rays. The mice received A/He spleen cells in doubling dilutions, beginning with a maximum dose of 60×10^6 cells per mouse. Each mouse in a set was given a different cell dose. Of 12 mice that received 15×10^6 A/He cells or more, all died within 42 days after cell injection. Of 15 mice that received 3.8×10^6 A/He cells or less, all survived the 120-day observation period. The minimum cell dose that produced GVH death was 15×10^6 cells per mouse in males and 7.5×10^6 cells per mouse in females.

The requirement for a sublethal dose of total-body x-rays in the GVH death of adult hybrid mice was studied. Five sets of six hybrid mice grouped according to age and sex were given total-body x-ray doses of between 50 and 500 R. Each mouse in a set received a different x-ray dose and each was given 12×10^6 A/He spleen cells. Of 15 mice that received 300 R or more, all died within 85 days after cell injection. Of 10 mice that received 100 R or less, all survived the 120-day observation period. The minimum x-ray dose that produced GVH death was 300 R in males and 200 R in females. The dependence of GVH death upon parent strain cell dose and x-ray dose is consistent with previous studies of the GVH response in adult hybrid mice.^{14,15}

The effect of anti-CBA ascitic fluid upon the GVH mortality of adult hybrid recipients of A/He spleen cells was examined (Table 2). Twenty-four mouse pairs were given x-ray doses of 360 R and cell doses of $8-64 \times 10^6$ cells per mouse. Test mice given IaCBA ascitic fluid died earlier than those given control serum or no serum ($P < 0.025$). The observation period was 30 days, and the mean of the differences in survival time was -2.54 days. Sixteen mouse pairs were given x-ray doses of 360 R and cell doses of $2-5 \times 10^6$ cells per mouse. Each of these pairs was of the same age and sex as a pair from the higher cell dose group described previously. They were housed with the corresponding higher dose pair, received a dilution of the same cell preparation given the higher dose pair, and were given ascitic fluid or serum at the same times, from the same aliquots, and in the same amounts as the higher dose pair. At the lower cell dose levels, the GVH response appeared later, and an observation period of 60 days was selected. The mean difference in survival time for the lower cell dose pairs was +3.00 days, indicating that the anti-CBA recipients may have been protected rather than harmed in this range of cell dosages, but this difference was not statistically significant ($0.1 < P < 0.2$).

Table 2
THE EFFECT OF ANTI-CBA ASCITIC FLUID ON GVH MORTALITY OF
(A/He x CBA) HYBRID ADULT MICE GIVEN
A/He SPLEEN CELLS*

No. of mouse pairs	$\bar{D}^\dagger$ (days)	P	A/He cell dose per mouse ($\times 10^{-6}$)	Observation period (days)
24	-2.54	<0.025	8-64	30
16	+3.00	0.1-0.2	2-5	60

* All mice were given 360 R of total-body x-rays 1 day before cell injection. The test mice received A/He spleen cells and IaCBA ascitic fluid. Control mice received A/He spleen cells and control serum or no serum. Ascitic fluid and serum injections were given i.p. in 0.5 ml amounts 1 day before and 3-4 days after cell injections. Survival of mice was followed for 30 or 60 days, starting with the injection of cells as day 0.

[†] $\bar{D}$ is the mean difference in survival times of standard and test mice.

The enhanced GVH mortality attributable to anti-CBA ascitic fluid in hybrid mice receiving higher doses of A/He spleen cells is consistent with the finding of increased spleen weight attributable to antihost alloantibody. The failure of GVH mortality to be enhanced by the same ascitic-fluid preparations in mice receiving lower doses of spleen cells indicates that the ascitic fluid did not exert a toxic effect in the absence of a particularly vigorous GVH reaction.

DISCUSSION

Splenomegaly in infant hybrid mice given parent strain lymphoid cells has been attributed to a GVH reaction.¹ When (A/He x CBA)F₁ infant mice were given A/He spleen cells, splenomegaly occurred as a consequence of a GVH reaction between the donor cells and the CBA components of the host tissues. Infant hybrid mice given A/He cells and an anti-CBA preparation had larger spleens than had mice given A/He cells and control serum. If the mice received hybrid cells instead of A/He cells, anti-CBA preparations had no effect on spleen weight. When a mixture of anti-CBA antiserum and hybrid spleen cells was injected, the antibody might have bound to the cells and thereby failed to cause spleen enlargement. However, although anti-CBA ascitic fluid injections and hybrid cell injections were given on different days, the former still failed to produce spleen enlargement. Thus the effect of the anti-CBA preparations was not a direct effect of antiserum on target cells, but required the presence of cells giving rise to a GVH reaction.

The active factor in the anti-CBA preparations could be removed specifically by absorption with CBA spleen cells. This provided strong evidence that the spleen-enlarging effect of antiserum was attributable to an antibody directed against CBA tissue antigens.

Increased vigor of the GVH response in mice given antihost alloantibodies has been described in the adult mortality model by Batchelor and Howard,⁸ who called this phenomenon "synergy." A synergistic effect in an adult mortality model also is indicated in an experiment previously described in which anti-CBA ascitic fluid was given to one member of each hybrid mouse pair receiving $8-64 \times 10^6$ A/He spleen cells. These data would be more straightforward if control mice had received nonimmune ascitic fluid rather than control serum. However, it is demonstrated herein that ascitic-fluid injections, when compared with control serum injections, do not alter spleen weight in hybrid cell recipients. Furthermore, enhanced mortality in anti-CBA recipients would be expected at lower spleen cell doses if the earlier deaths were attributable to a toxin which acted independently of the GVH response; however, adult anti-CBA ascitic fluid recipients given $2-5 \times 10^6$ spleen cells did not die before controls. Apparently a minimum cell dose is required for synergy.

Mortality assays for synergy may be criticized, since direct tissue damage by the antibody alone might explain increased mortality. The spleen weight model avoids this difficulty by measuring the proliferation of the responding cell populations. Identical alloantisera, cell preparations, and strain combinations gave a synergistic effect in both the adult mortality and spleen weight models. Hence, the mechanism of synergy in both models was probably the same. A lymphoproliferative response classically occurs early in the GVH response.^{1,2} Earlier deaths in adult mice receiving antihost antibodies may have resulted from a more vigorous proliferative phase of the GVH response.

The synergistic effect of anti-CBA ascitic fluid was noted in the spleen weight model with CBA spleen cells, as well as with A/He spleen cells. Failure to prevent the GVH response by giving antibody against the CBA parental graft may be attributable to timing of antibody injection.

tions relative to cell injections. When A/He spleen cells were given with A/He anti-CBA allo-antiserum, both cells and antiserum were reactive against the CBA components of the F_1 recipient; when CBA cells were given, the parental cells were reactive against the A/He component and the antiserum was reactive against the CBA component. Hence, synergy occurred with antibodies and cells directed against different antigenic determinants. This result is in sharp contrast to the tumor enhancement studies of Möller, in which antibody was required against all the foreign antigens of the graft to prevent tumor rejection.¹⁷

Several reports have described mitigation of the GVH response by passively administered antihost antibody.⁷⁻¹⁰ Batchelor and Howard report both synergy and antagonism of the GVH response in different mouse strains under similar conditions of cell and antiserum dosage.⁸ Choice of host, graft, and alloantiserum determines the three major elements of this response. When Voisin and Kinsky gave A strain newborn mice CBA cells and CBA anti-A serum, they found antagonism of the GVH response.⁷ In contrast, data presented in this study show that synergy occurred when CBA cells and A/He anti-CBA serum were given to hybrid hosts. The CBA cells supported either synergy or antagonism of the GVH response. Batchelor and Howard have described a pair of experiments in adult $(A \times C57BL)F_1$ mice.⁸ An anti-C57BL serum and A strain cells acted synergistically, but C57BL anti-A serum was antagonistic with C57BL cells. The hybrid host supported either synergy or antagonism. In all reported experiments, the synergistic serum was produced in A strain mice. This suggests that the mouse strain chosen for alloantiserum production may be important in determining whether a synergistic or antagonistic effect is obtained.

Mouse strains differ in their response to soluble antigens and to erythrocyte alloantigens.¹⁸⁻²² The A strain has been shown to produce a greater humoral immune response than does the CBA to keyhole limpet hemocyanin¹⁸ and to several hapten-protein conjugates.²² The magnitude of an immune response, however, is not determined by the mouse strain alone. Vaz and Levine compared the responses of A/He and SWR mice to hapten conjugates of bovine γ -globulin and bovine pancreatic ribonuclease. They found that A/He mice developed higher titers to the former antigen, and SWR mice produced higher titers to the latter.²² The antibody response is therefore determined by the particular combination of antigen and mouse strain.

Alloantigens may play a role similar to that of soluble antigens in determining the type of humoral response. C57BL anti-CBA serum acted synergistically,⁸ as did A/He anti-CBA in the experiments which have been described; C57BL anti-A acted antagonistically,⁸ as did CBA anti-A.⁷ In these instances, the alloantigen rather than the immunized mouse strain appears to determine the effect of antiserum on the GVH response.

Mouse strains differ qualitatively as well as quantitatively in their response to soluble antigens. Coe has shown that A strain mice produce both 7S γ_1 and 7S γ_2 anti-hen egg albumin under conditions that elicit only 7S γ_1 production in CBA mice. Production of 7S γ_2 antibody could be induced in CBA mice with higher doses of hen egg albumin, by addition of Freund's complete adjuvant, or by use of a different antigen.¹⁹ Mouse strains might be expected to differ also in their immunoglobulin class response to alloantigens.

Differences in antibody class response to alloantigens may explain the contrasting effects of different alloantisera on the GVH response. It is well known that different immunoglobulin classes are associated with different biological properties.^{23,24} The classes of alloantibody used in passive immunization of the host may determine whether the GVH response is magnified or reduced.

A particular antigenic stimulus may simultaneously elicit several antibody classes; synergism and antagonism of the GVH response may exist in competition within a host. Separation of antibody classes by physical-chemical or immunochemical methods may separate synergistic from antagonistic antibody. An analogous separation of cytotoxic and tumor-enhancing antibodies from a single serum pool has been reported by Voisin et al.²⁵

The GVH reaction is usually considered to be a cell-mediated immune response, but the grafted cells may also produce antibody. Antibodies may not be detected because of absorption by host components. The observation that the GVH response may be modified by passive immunization with antihost antisera suggests that antibody production by grafted cells may in part determine the magnitude of the response. Production of particular classes of antihost antibody by donor cells in a GVH response may explain why different strain combinations have different cell dose requirements. Production of synergistic antibodies might lower the dose of parent strain cells required to develop a GVH response; production of antagonistic antibodies might increase the required cell dose.

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ANTIGEN TRAPPING IN THE LAMINA PROPRIA AND PRODUCTION OF IgA ANTIBODY*

By

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IgA antibody is found predominantly in the external secretions of the respiratory and gastrointestinal tracts. It is thought to be responsible for protection against some infections of the mucous surfaces of the body.¹ Much of it is produced by plasma cells in the lamina propria of mucous membranes. This paper describes a specialized antigen-trapping mechanism in the lamina propria of the major bronchi and the small intestine. Circumstantial evidence suggests that antigen retained by these cells is responsible for the production of IgA antibody in the lamina propria.

MATERIALS AND METHODS

Salmonella typhi (Ty2w) flagella were prepared by the method of Kobayashi et al.² Hen egg albumin (HEA), 5 times recrystallized, was purchased from Pentex Inc., Kankakee, Ill. Both proteins were labeled with radioiodine, ¹²⁵I, to a specific activity of 5 mCi/mg and a substitution rate of about one atom of iodine per protein molecule by our modification of the Chloramine-T procedure.³ Carrier-free iodine-125 was obtained from New England Nuclear Corporation, Boston, Mass. Adult male Sprague-Dawley rats were obtained from Sprague-Dawley Inc., Madison, Wisc., and BALB/c mice (BALB/cAnN) from the Animal Production Section, Laboratory Aids Branch, National Institutes of Health, Bethesda, Md. All were fed standard laboratory diet and water ad libitum. One week before injection of ¹²⁵I-labeled protein, Lugol's iodine solution was added to the drinking water to insure rapid excretion of free radioiodine. Organs fixed in Tellyesnicky's fluid were embedded in paraffin and sectioned for microscopic examination by standard procedures. Autoradiographs were made by dipping microscopic sections in Kodak NTB2 nuclear track emulsion. After two months they were developed with Kodak Dektol developer, and stained with nuclear fast red. Titanium dioxide (TiPure R900, E. I. du Pont de Nemours and Co., Wilmington, Del.) is an excellent inert particle for studies of phagocytosis. It was used as described elsewhere.⁴

RESULTS

Two groups of rats, one normal and one immunized three weeks earlier with flagella, were injected intravenously with 10 µg of ¹²⁵I-labeled flagella. Some of the animals were injected simultaneously with 100 mg of titanium dioxide. At selected intervals from 15 min. to 3 days after injection, the lungs, liver, and spleen were removed for histologic and autoradiographic studies. Antigen label was found in the lamina propria of the major bronchi in every animal. It was retained in association with reticular cells and capillary endothelial cells (Figure 1, A and B).

* This report is taken from a paper that appeared in the Journal of the Reticuloendothelial Society, 11:245, 1972.

Figure 1. Autoradiographs of ^{125}I -labeled flagella localization in the lamina propria. A. This section of a rat bronchus removed 15 min. after intravenous injection of $10\ \mu\text{g}$ of ^{125}I -labeled flagella shows antigen label in the lamina propria. X30. B. This antigen persists in the bronchus at 2 days after injection. Silver grains of the autoradiographs are seen over capillary endothelial cells and reticular cells. Bronchial mucosa is seen on the right, and cartilage on the left side. X480. C. The small intestine of a BALB/c mouse shows localization of the same antigen in the lamina propria at 30 min. after intravenous injection of $5\ \mu\text{g}$. X120. D. At 2 days after injection, the antigen persists in the mouse intestine. X240.

None was seen in the lymphoid or plasma cells in the area. The amount retained in the lamina propria varied greatly from animal to animal and diminished with time after injection. There was no apparent relationship between the amount retained in the lamina propria and the amount retained in the dendritic reticular cells found in lymphoid follicles of the spleen or red pulp

macrophages in the spleen or the Kupffer cells in the liver. Previously immunized animals usually retained less antigen in the lamina propria than did normal animals. Titanium dioxide was found in many types of macrophages in the lung, spleen, liver, adrenal glands, lymph nodes and thymus, but not in the lamina propria.

In another experiment, we studied the localization of ^{125}I -labeled flagella in the lamina propria of the small intestine of BALB/c mice. The mice were injected intravenously with 5 μg of ^{125}I -labeled flagella. The spleens, livers and small intestines were removed at 30 min. and 2 days after injection for autoradiographic examination. The antigen was found localized in the lamina propria of the small intestine in a pattern similar to that seen in the bronchi of rats (Figure 1, C and D). A high concentration of antigen label was found in the lamina propria at 30 min., and a lower concentration at 2 days after injection. Previously immunized animals usually retained less antigen label than normal ones at both times. The label was retained by the stromal cells of the lamina propria in a pattern similar to that seen in the rat bronchi. The antigen was found in areas crowded with lymphoid and plasma cells, but little, if any, could be seen in these cells.

In a third experiment, both normal and immunized Sprague-Dawley rats and BALB/c mice were injected intravenously with ^{125}I -labeled HEA in doses of 100 μg and 25 μg , respectively. At 30 min., 2 days, and 5 days after injection, the antigen label was found in many types of phagocytic cells in the spleen, liver, lungs, and bone marrow, but not in any of the cells of the lamina propria.

DISCUSSION

We have described an antigen-trapping mechanism in the lamina propria of bronchi and small intestine. The cells in the lamina propria which retain antigen are different from other types of phagocytic cells in that they select the type of material that is localized. Radioiodinated *S. typhi* flagella are the only material that we have been able to demonstrate in these cells. Titanium dioxide and ^{125}I -labeled HEA are readily taken up by many types of phagocytic cells in other parts of the body, but are not localized in the lamina propria. In an extensive review of human and animal material from our pathology slide library and previous experiments, we have found a wide variety of particles and pigments localized in phagocytic cells in many organs, but we have not been able to demonstrate the localization of any pigments or phagocytosed material in the lamina propria of the small intestines or bronchi.

The lamina propria contains a stroma of reticular cells and argyrophile fibers similar to those of lymphoid tissue.⁵ The antigen is retained in close association with this stroma. Enmeshed in these fibers are many lymphoid and plasma cells which are involved in the synthesis of antibody, especially IgA antibody.¹ *Salmonella typhi* is an unusual antigen in that it has been reported to stimulate production of secretory IgA antibody by the intestine following parenteral injection.¹ To our knowledge, the only other antigen known to stimulate the production of secretory IgA antibody after a single parenteral injection is the cholera vibrio.¹ The close association of the antigen-retaining cells with the IgA antibody-forming cells has led us to suggest that the antigen retained in the lamina propria is responsible for stimulating IgA antibody production. We do not know how antigen is trapped in the lamina propria, but we can formulate a reasonable hypothesis based on the behavior of other phagocytic mechanisms. It seems to be a general rule that phagocytosis requires the interaction of the phagocytosed particle with an opsonin. This op-

Figure 2. Autoradiographs of ^{125}I -labeled flagella localized in the three types of phagocytic cells have been tentatively associated with the production of antibody of the IgA (A), IgM (B), and IgG (C) classes. A. This higher magnification of Figure 1A shows antigen localization in the lamina propria. X200. B. Antigen is retained in the red pulp of the spleen at 5 min. after intravenous injection of $10\ \mu\text{g}$ of flagella into a rat immunized intravenously 3 weeks earlier with $10\ \mu\text{g}$ of unlabeled flagella. X60. C. Antigen is retained in the dendritic reticular cells of the spleen of a rat immunized in the same way as animal B and splenectomized at 2 days. X60.

sonin may be either specific in the form of a natural or immune antibody, or it may be nonspecific.⁶ Since many enteric organisms have cross-reacting antigens, we should expect to find that animals produce a secretory antibody that is at least weakly reactive with our flagella. In addition, "natural antibodies" against many bacteria including Salmonella typhi have been reported many times.⁷ We suggest that animals have some sort of natural or cross-reacting opsonin to typhi flagella in their lamina propria, and that this opsonin promotes the uptake of antigen by the reticular and endothelial cells. The unusual ability of typhi and cholera to stimulate secretory IgA antibody may be related to the presence of opsonins for these antigens in the lamina propria.

The production of two other classes of antibody, IgG and IgM, has been associated with antigen localization and cell proliferation in specific areas of lymphoid tissue (Figure 2). There is abundant evidence that the production of IgG antibody is associated with antigen retained by the dendritic reticular cells located in the lymphoid follicles of the spleen and lymph nodes.⁸ Studies with radiation⁹ and drug-induced¹⁰ suppression of antibody formation, the ontogeny¹¹ and phylogeny¹² of immune responses, and histologic localization of antibody-forming cells¹³ all demonstrate a relationship between either lymphoid follicle proliferation or dendritic reticular cell activity and the production of IgG antibody. The production of IgM antibody in the spleen takes place largely, if not entirely, in the red pulp.¹³ Neither dendritic reticular cells nor any recognizable white pulp structures are necessary for the synthesis of IgM antibody.^{11,12} Howard et al.¹⁴ showed that lipopolysaccharide antigens which usually stimulate only IgM antibody are retained only in the red pulp of the spleen. In addition, Mosier¹⁵ and Pierce¹⁶ have shown that macrophages which adhere to plastic are necessary for the induction of IgM antibody formation in vitro. The dendritic reticular cells labeled with ¹²⁵I flagella do not detectably adhere to plastic (unpublished observation). The antigen-containing cells that stimulate IgM antibody production in the spleen have not been identified with certainty, but we can be quite sure that they are different from either the dendritic reticular cells or the antigen-retaining cells in the lamina propria.

The implication of these experiments seems clear: the localization of antigen in lymphoid tissue is a major factor in determining the type of antibody produced. Three classes of antibody, IgA, IgG, and IgM, have been associated with antigen localization in specific areas of lymphoid tissue. It is reasonable to expect that the production of the other classes of antibody may also be associated with antigen localization in particular types of phagocytic cells. Further investigation of the factors that control antigen localization in different types of phagocytic cells should add significantly to our knowledge of the mechanisms that determine the type of immune response produced by a given antigenic stimulation.

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