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ALTERATIONS IN DEOXYRIBONUCLEIC ACID-BOUND AMINO ACIDS AFTER THE ADMINISTRATION OF DEOXYRIBONUCLEIC ACID-BINDING DRUGS

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Abstract—DNA was isolated from liver, kidney, spleen and small intestines of rats treated with high doses of actinomycin D, proflavin, nitrogen mustard (HN2) and mitomycin C. The DNA-associated amino acids differed profoundly in relative and absolute amounts. Treatment *in vitro* of liver homogenate with one of these drugs (proflavin) did not produce this or any change in the amino acids bound to DNA, which suggests a complex interaction. The changes are not random, since they: (1) are qualitatively and quantitatively reproducible; (2) include both additions and deletions of amino acids; and (3) include an extra ninhydrin-positive component associated with the aromatic amino acids but not found in the residual host proteins. The new amino acid is apparently the same one previously found in certain tumor DNA's. Despite the differences in molecular structure, reported pharmacologic effects, and nature of their interactions with DNA of the four compounds studied, they all produced profound changes in the amount and kind of amino acids bound to DNA so as to suggest that this may be a significant factor in the mechanisms of their action.

THE ANTIBIOTICS, actinomycin D[1–4] and mitomycin C,[5–8] as well as synthetic compounds such as proflavin (3,6-diaminoacridine)[9–12] and the bifunctional nitrogen mustard, HN2 (*N*-methyl-bis[2-chloroethyl]amine HCl),[13–15] which alkylate or bind to DNA, have been shown to have pronounced chemical effects both in whole animals and in isolated enzyme systems. The first three compounds combine with DNA, distorting its structure to an extent measurable by various physical parameters[2, 4, 8, 16] and hence interfering with its function. The complexes formed with DNA are characteristic of the particular type of compound, i.e. hydrogen bonding of actinomycin D to the double helical DNA,[2, 4] mono- and bifunctional alkylation of the guanine moiety by mitomycin C,[8] and intercalation of proflavin in the double helical structure.[16, 17] Recent studies on the kinetics of complex formation by N-7 substituted actinomycin, the hydrodynamic properties of actinomycin D complexes with low molecular weight DNA,[18] and the flow dichroism of mitomycin C–DNA complex[19] seemed to implicate intercalation as an additional mode of binding for these antibiotics. In re-evaluating these data along with those reported for polycyclic hydrocarbons,[20] Waring[21] has suggested that intercalation may be a process common to a majority of the compounds which bind DNA.

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Although mono- and bifunctional alkylations are involved in the interaction of HN2 and DNA, there appears to be little, if any, change detectable in the physical properties of the DNA isolated from systems treated with the compound *in vivo*.[14, 15, 22, 23] The incidence of interstrand crosslinks is extremely low, about one per 23×10^6 daltons[24] in contrast to one per 10^6–10^7 daltons from systems treated with mitomycin C.[8] However, there is an increase in the amount of protein which is bound to DNA.[25–27] This bound protein is resistant to the usual phenol-detergent or salt separations.[22] The amount of DNA which is extractable by standard procedures also decreases,[22] reminiscent of that observed in irradiated bacterial cultures.[28]

The well documented persistence of amino acid residues in DNA isolated from a variety of sources has been reviewed.[29–31] Although preliminary data from sequential treatment with deoxyribonuclease and dinitrofluorobenzene* indicate that these residues are present as peptides or small proteins, they are determined only after hydrolysis of DNA and consequently are designated as amino acids. The stability of these components to dialysis (an integral part of the isolation procedure) and their behavior on banding in cesium chloride gradient and gel filtration after treatment with 5 M urea[31] are reminiscent of certain dyes and antibiotics.[32] The variations of these amino acids in bacterial DNA with culture medium[31, 33] and growth phase[31] and in tumor DNA with successive transplantation of experimentally induced tumors[34] suggest that they may play some unique role in cellular function. It is conceivable that such amino acid residues could be displaced or modified *in vivo* by the presence of compounds capable of complexing with DNA and distorting its structure and function. The four compounds reviewed above have been studied to test the tenability of this suggestion. The results are presented in this paper.

EXPERIMENTAL

Mitomycin C was obtained from Bristol Laboratories (Syracuse, N.Y.); the other three compounds, HN2 (Mustargen), actinomycin D (Lyovac Cosmegen) and proflavin dihydrochloride, were obtained from Merck, Sharpe & Dohme (Rahway, N. J.). Actinomycin D and HN2 were reconstituted by adding sterile water. Mitomycin C and proflavin were dissolved in 0·1 M sodium phosphate buffer, pH 7·2.

Fourteen-week-old male CFE rats (250–320 g), obtained from Carworth Farms (New City, N. Y.), were maintained on a diet of Purina laboratory chow and water *ad lib*. There were fourteen animals in each experimental group. The compounds were administered by intraperitoneal injections. Treatment consisted of three daily doses of about one-half LD_{50}; i.e. 2 mg HN2/kg/day,[35] 1 mg mitomycin C/kg/day,[5] and 0·1 mg actinomycin D/kg/day.[1] On the fourth day, each animal was given a single injection containing 20 times the daily dose and sacrificed after 1 hr. Another group of animals were given a single dose of twice LD_{50} of mitomycin C and sacrificed after 2 hr. To maintain the volume injected in the range of the above compounds, a saturated solution of proflavin was used. The actual concentration was determined spectrally at 261 and 444 mμ. The daily regimen consisted of 20 mg/kg/day while the final dose was ten times this concentration. The multidose regimen had been chosen to insure a systemic response to sublethal levels of the drug before the final short-term lethal dose. Other studies in our laboratories† indicate that such treatment produced morphologic

* J. S. Salser and M. E. Balis, unpublished observations.
† J. G. Cappuccino and M. E. Balis, unpublished observations.

changes similar to those observed with the usual 5- to 7-day regimen at LD_{50}. Control animals were injected with volumes of phosphate buffer equivalent to that used for drugs.

Random samples of the tissues were removed from each experimental group for microscopic examination. All the remaining tissues were frozen with solid CO_2 immediately after removal from the animals and subsequently pooled for DNA isolation. Control livers, however, were divided into three groups, namely: (1) proflavin added during homogenization to give a final concentration of 2 mg/ml of homogenate; (2) ^{14}C-phenylalanine, uniformly labeled (New England Nuclear, Boston, Mass.; $7{\cdot}4 \times 10^8$ cpm/μmole) added to give 10^5 cpm/ml of homogenate; and (3) no addition. All three samples were stirred gently for 15 min at room temperature before addition of aqueous phenol. The final deproteinized DNA from the first two samples was checked spectrally for proflavin or assayed for ^{14}C-phenylalanine using a Packard Tri-Carb liquid scintillation counter.

The isolation, characterization and hydrolysis of DNA as well as the methods for the analysis and calculation of the amino acid residues have been described in detail.[29, 30]

RESULTS

During the multidose regimen, animals showed weight losses ranging from 10–15 per cent of the initial body weight. Microscopic examination of the tissues showed characteristic morphologic changes attributed to multiple and lethal doses of these compounds.[1, 4, 12, 25, 35]

The results presented in this paper do not include either tryptophane or glycine.[29] The values for glutamic acid and aspartic acid include their respective amides, which are hydrolyzed under the present experimental conditions. The values of the individual amino acids are expressed as mole per cent of the sum of the amino acid residues experimentally determined. The latter, designated as total amino acid residues, are given in micromoles per 100 mg of DNA. The amino acids have also been grouped as basics, acidics and neutrals; the mole per cent of each of these groups has been included in the tables. The designation, X-DNA, where X is the compound, has been used merely for convenience in identifying the sample and does not imply bound compound. Values from a separate experiment,* using untreated 13-week-old animals of the same weight range, have been included as untreated controls (NT) in the tables.

There was a remarkable agreement in the results obtained from three replicate isolations of control liver DNA (Table 1). Addition of proflavin or labeled amino acid during the isolation procedure did not change either the composition or the total amount of amino acid residues. The crude DNA initially isolated from the liver sample containing added proflavin formed a spool of fibers on the stirring rod much more readily than the usual liver DNA samples. However, no proflavin could be detected spectrally in the repeatedly deproteinized DNA, suggesting that if proflavin had been bound to DNA *in vitro*, it was lost during the subsequent purification steps. In the sample with added ^{14}C-phenylalanine, no label ($< 0{\cdot}5$ cpm/mg DNA) could be detected in the final isolated DNA. It appears that the presence in the homogenate of free amino acids or compounds known to bind DNA resulted in neither indiscriminate association of these components with DNA nor their random adsorption on the

* J. S. Salser and M. E. Balis, unpublished observations.

polynucleotide. These data lend further credence to the existence of these amino acid residues as biologically significant entities.[29]

TABLE 1. REPLICATE ISOLATIONS OF CONTROL LIVER DNA*

Amino acids	Standard conditions	Proflavin	^{14}C-phenylalanine
Lysine	18·2	18·3	18·1
Histidine	1·1	1·0	0·9
Arginine	1·6	2·0	1·6
Aspartic acid	29·1	28·3	30·3
Glutamic acid	8·1	7·7	7·4
Threonine	5·1	5·5	4·9
Serine	8·2	7·7	8·3
Proline	1·1	0·8	1·0
Alanine	6·0	6·3	5·9
Half cystine	4·9	5·5	5·1
Valine	4·2	3·7	4·3
Methionine	1·4	1·4	1·3
Isoleucine	2·5	2·3	2·2
Leucine	4·4	4·7	4·7
Tyrosine	1·8	2·1	1·9
Phenylalanine	2·2	2·4	2·0
Basic	20·9	21·3	20·6
Acidic	37·1	36·0	37·7
Neutral	42·0	42·7	41·7
Total (μmoles/100 mg DNA)	1·200	1·195	1·245

* Pooled livers from fourteen male CFE rats were divided into three equal parts. DNA was isolated as previously described[29, 30] (standard conditions) and with the addition of either proflavin (2 mg/ml homogenate) or uniformly labeled ^{14}C-phenylalanine (10^5 cpm/ml homogenate) during the homogenizations. All three samples were stirred for 15 min before addition of aqueous phenol. Unless otherwise specified, values are expressed as mole per cent, calculated from the total amino acids determined.

Although there was a difference in age (1 week) and treatment, there were only minor variations in the amino acids (lysine and aspartic) associated with the two sets of control liver DNA (Table 2). Actinomycin D appeared to be the only compound causing an appreciable increase in the total amounts of these residues; there were about 4·5 times as much as the controls. Relatively small changes were observed after treatment with proflavin and the two alkylating agents, HN2 and mitomycin C. The total amounts were 80 and 150 per cent, respectively, of the control values. Of all the liver samples studied, only the crude DNA from proflavin-treated animals formed fibers readily during the precipitation step. This tendency was even more pronounced than when proflavin was added to control liver during the isolation procedure (*cf.* Table 1).

There appeared to be considerable variation in the relative distribution of the individual amino acid residues. An examination of the mole per cent of the basics, acidics and neutrals indicated that treatment with HN2 resulted in an increase in basics, primarily arginine, and a decrease in acidics, which was mostly attributable to aspartic acid. Although there was no apparent change in the total neutrals, there were variations in most of the amino acids; exceptions included alanine, methionine and the aromatic amino acids. With the other three compounds, there was a simultaneous decrease in basics and acidics, resulting in a rather large increase of total neutrals. As would be expected from such an observation, the more dramatic changes are to be found in lysine, aspartic and glutamic acid. The relative distribution of the neutral

TABLE 2. EFFECT OF COMPOUNDS WHICH BIND DNA ON AMINO ACIDS ASSOCIATED WITH LIVER DNA*

Amino acids	Control		Treated				
			HN2‡	Mitomycin C		Act D'§	Proflavin
	NT	PO_4† buffer		Single	Multidose		
Lysine	20·3	18·2	20·7	5·4	5·8	6·1	4·8
Histidine	0·3	1·0	0·9	2·6	2·4	1·5	1·3
Arginine	1·5	1·7	24·2	4·4	5·0	3·7	4·1
Aspartic acid	32·6	29·2	5·7	11·6	11·3	12·6	11·6
Glutamic acid	7·4	7·7	5·6	15·1	13·5	14·6	11·6
Threonine	4·0	5·2	2·6	4·0	4·2	5·7	4·6
Serine	8·2	8·1	5·1	11·5	11·9	6·2	6·7
Proline	+	1·0	3·3	4·2	3·9	5·9	6·5
Alanine	5·6	6·0	5·9	6·5	6·3	10·4	10·4
Half cystine	5·1	5·2	8·4	10·6	11·4	1·0	5·9
Valine	3·2	4·0	8·1	7·8	7·7	9·0	19·4
Methionine	1·0	1·4	1·5	0·9	0·8	1·5	0·6
Isoleucine	2·3	2·3	1·6	2·7	2·8	4·7	2·1
Leucine	4·5	4·5	3·2	6·3	6·3	10·4	4·9
Tyrosine	1·9	1·9	1·2	3·0	2·9	2·7	2·2
Phenylalanine	2·0	2·2	1·7	3·4	3·7	4·1	3·4
Basic	22·1	20·9	45·8	12·4	13·2	11·3	10·2
Acidic	40·0	36·9	11·3	26·7	24·8	27·2	23·2
Neutral	37·9	42·2	42·9	60·9	62·0	62·0	66·6
Total (μmoles/100 mg DNA)	1·256	1·213	1·730	2·078	2·051	5·896	0·921

* Each sample contained pooled tissue from fourteen male CFE rats. Details of the dose regimen are given in Experimental, and those of DNA isolation and amino acid analysis, in earlier studies.[29, 30] Abbreviations used include: NT, no treatment; HN2, nitrogen mustard; Act D, actinomycin D. Unless otherwise specified, values are expressed as mole per cent, calculated from the total amino acids determined. The plus sign (+) designates presence in trace amounts.

† Average of three replicate isolations (*cf.* Table 1) with variations of ±5 per cent.

‡ Average of two replicate isolations with variations of ±3 per cent.

§ Neither methylvaline nor sarcosine was present in these hydrolysates.

amino acids varied with the compound used; the different mitomycin C regimen however, had no effect on either the total or the relative amounts of these residues. With the antibiotics, there were changes in serine, proline, valine, half cystine, leucine and alanine (only with actinomycin D); with proflavin, the changes involved serine proline, alanine and valine.

In kidney DNA (Table 3), there were slight variations in lysine and perhaps valine in the controls. The total amounts increased with treatment; the most pronounced increases were those observed with HN2 (3·5 times the controls) and actinomycin D (almost 3 times the controls). Unlike liver DNA, the single dose of mitomycin C resulted in a greater increase than did the multiple dose, and proflavin caused an increase rather than a decrease. In HN2-DNA, there was an increase in basics, reflecting the small increments of each of the three amino acids. The relative distribution of the acidics remained unchanged, with decreases appearing in the neutrals, notably in serine and alanine. After treatment with mitomycin C and actinomycin D, there were only minor variations in the relative distribution of the individual amino acids. Nonetheless, the changes with a single dose of mitomycin C involved alanine, phenylalanine and leucine, whereas those with multiple doses involved serine, valine and leucine. In spite of the much larger total amounts, the

TABLE 3. EFFECT OF COMPOUNDS WHICH BIND DNA ON AMINO ACIDS ASSOCIATED WITH KIDNEY DNA*

Amino acids	Control		Treated				
			HN2	Mitomycin C		Act D†	Proflavin
	NT	PO_4 buffer		Single	Multidose		
Lysine	5·5	7·4	12·4	4·3	5·5	8·1	6·2
Histidine	1·6	1·8	3·9	1·8	1·7	1·9	1·6
Arginine	4·6	4·0	5·7	5·3	5·8	5·4	4·4
Aspartic acid	10·6	10·5	9·9	11·6	11·1	11·4	11·7
Glutamic acid	12·9	12·7	12·1	14·3	11·6	12·4	14·2
Threonine	5·7	5·1	5·1	5·5	5·2	5·7	5·3
Serine	10·6	10·4	6·6	10·2	7·5	6·5	9·3
Proline	6·7	6·4	6·3	5·7	7·4	5·4	6·0
Alanine	9·8	9·9	7·6	7·8	8·6	9·3	9·5
Half cystine	1·9	2·0	+	1·5	2·5	1·0	2·9
Valine	8·9	7·9	7·2	7·1	10·3	8·3	9·7
Methionine	1·2	1·3	1·6	1·8	1·6	1·3	1·3
Isoleucine	3·8	3·6	4·4	4·0	4·3	4·6	2·9
Leucine	8·0	8·4	9·3	9·5	9·1	10·3	7·3
Tyrosine	3·5	3·4	3·3	3·0	2·8	3·2	2·8
Phenylalanine	4·6	5·1	4·6	6·6	4·9	5·2	5·0
Basic	11·7	13·2	22·0	11·4	13·0	15·4	12·2
Acidic	23·5	23·2	22·0	25·9	22·7	23·8	25·9
Neutral	64·8	63·6	56·0	62·7	64·3	60·8	61·9
Total (μmoles/100 mg DNA)	3·751	3·679	13·410	5·686	4·095	9·946	4·161

* Each sample contained pooled tissues from fourteen male CFE rats. Details of the dose regimen are given in Experimental, and those of DNA isolation and amino acid analysis, in earlier studies.[29, 30] Abbreviations used include: NT, no treatment; HN2, nitrogen mustard; Act D, actinomycin D. Unless otherwise specified, values are expressed as mole per cent, calculated from the total amino acids determined. The plus sign (+) designates presence in trace amounts.

† Neither methylvaline nor sarcosine was present in these hydrolysates.

effects of actinomycin D were not too different from those of multiple-dose mitomycin C, i.e. involving serine and leucine. There appeared to be very little change in the individual amino acids associated with proflavin-DNA. The results summarized here are distinctly different from those of liver DNA, tending to emphasize the variations of the amino acid residues with tissue[29] as well as the variations in tissue response to a particular drug.

The considerable variations in composition of the control spleens (Table 4) were not unexpected in view of the differences in spleen size (untreated or NT spleens were smaller) and the long recognized responses of this tissue to external stresses. The differences observed in the two sets of controls could be the consequence of the physiological state of the tissue. The total residues increased with treatment: about five times control values in HN2-DNA, three times in proflavin-DNA, and 1·5–2·0 times in antibiotic-DNA. The single dose of mitomycin C resulted in a greater increase than did the multiple dose, just as in kidney DNA.

Since the variations in the controls involved all the basic amino acids, assessment of changes in this particular group was rather difficult. For example, the variations in histidine and arginine in the samples were no greater than those found between the two sets of controls. The only significant changes in the individual amino acids were the increases: (1) in aspartic, glutamic and perhaps isoleucine in HN2-DNA; (2) in

TABLE 4. EFFECT OF COMPOUNDS WHICH BIND DNA ON AMINO ACIDS ASSOCIATED WITH SPLEEN DNA*

Amino acids 1	Control		Treated				
			HN2	Mitomycin C		Act D†	Proflavin
	NT	PO_4 buffer		Single	Multidose		
Lysine	7·3	6·0	6·9	9·8	5·3	5·2	8·2
Histidine	8·1	4·0	2·9	2·4	2·5	1·4	1·8
Arginine	6·2	4·8	6·4	4·2	3·0	4·3	5·9
Aspartic acid	10·4	10·5	13·1	11·4	9·4	10·5	11·1
Glutamic acid	13·7	13·2	16·3	14·5	12·9	13·2	14·8
Threonine	4·6	6·6	5·7	5·7	6·6	5·3	5·8
Serine	9·8	7·7	7·5	10·6	13·1	7·6	7·8
Proline	6·0	5·9	5·4	6·4	5·2	4·7	5·2
Alanine	7·1	9·1	7·9	8·0	9·6	11·5	8·7
Half cystine	1·0	2·9	0·6	1·3	2·7	3·1	1·5
Valine	8·1	9·8	6·8	6·8	9·7	13·8	8·2
Methionine	1·0	1·1	1·3	1·2	1·9	1·0	1·2
Isoleucine	3·2	3·5	4·5	3·2	3·5	3·6	4·0
Leucine	7·5	8·0	7·7	7·7	8·2	8·4	9·4
Tyrosine	3·0	3·3	3·1	3·0	3·2	2·7	2·7
Phenylalanine	3·0	3·6	3·8	3·8	3·2	3·7	3·6
Basic	21·6	14·8	16·2	16·4	10·8	10·9	15·9
Acidic	24·1	23·7	29·4	25·9	22·3	23·7	25·9
Neutral	54·3	61·5	54·4	57·7	66·9	65·4	58·2
Total (μmoles/100 mg DNA)	3·148	2·747	14·446	5·252	4·459	5·041	8·295

* Each sample contained pooled tissues from fourteen male CFE rats. Details of the dose regimen are given in Experimental, and those of DNA isolation and amino acid analysis, in earlier studies.[29, 30] Abbreviations used include: NT, no treatment; HN2, nitrogen mustard; Act D, actinomycin D. Unless otherwise specified, values are expressed as mole per cent, calculated from the total amino acids determined.

† Neither methylvaline nor sarcosine was present in these hydrolysates.

lysine and glutamic in mitomycin C-DNA, single dose, and in serine, multiple dose; (3) in valine in actinomycin D-DNA; and (4) in glutamic in proflavin-DNA.

The DNA isolated from small intestines of animals treated with these compounds differed from those of the other tissues in that the largest increase in total residues was relatively small (80 per cent for HN2-DNA) and the decreases were larger than the one instance of 20 per cent found in liver proflavin-DNA (40–60 per cent for mitomycin C-DNA; Table 5). The effect of a single dose of mitomycin C was again greater than that of the multiple dose (*cf.* Tables 3 and 4). There may be a slight decrease in these residues in actinomycin D-DNA. The two sets of controls show a much closer agreement than any of the other tissues.

Some of the changes in the relative distribution were found in all the treated samples. There were increases in serine and, to a lesser extent, in proline as well as decreases in the acidics, leucine, isoleucine and alanine. The only exception was alanine, which was unchanged in the DNA from animals given a single dose of mitomycin C. The unique change was that of the aromatic amino acids, which appeared as single unresolvable peaks whose values were calculated as previously described.[30] The values ranged from 1·6 to 3·3 times that of the sum of the aromatic amino acids in the controls. The maximum effect was produced by either a single dose of mitomycin C or actinomycin D.

TABLE 5. EFFECT OF COMPOUNDS WHICH BIND DNA ON AMINO ACIDS ASSOCIATED WITH INTESTINAL DNA*

Amino acids	Control		Treated				
			HN2	Mitomycin C		Act D†	Proflavin
	NT	PO_4 buffer		Single	Multidose		
Lysine	6·5	6·2	10·5	3·2	3·5	1·6	4·5
Histidine	0·2	0·5	3·5	1·4	1·1	1·2	6·0
Arginine	6·1	5·8	9·0	4·5	9·3	5·8	4·3
Aspartic acid	15·3	15·1	8·0	7·4	8·0	7·0	7·3
Glutamic acid	12·8	12·7	11·0	9·4	7·1	6·5	6·7
Threonine	14·7	14·5	8·6	13·9	13·6	23·3	17·3
Serine	13·1	12·9	17·7	19·7	17·4	15·5	23·0
Proline	4·1	4·0	6·2	6·7	8·9	9·8	7·5
Alanine	7·1	7·2	5·1	7·8	5·5	5·7	5·9
Half cystine	+	0·9	2·3	1·4	1·8	0·6	0·8
Valine	5·4	5·6	5·1	6·3	7·8	5·6	4·5
Methionine	0·6	0·5	0·3	0·7	0·8	0·4	0·2
Isoleucine	3·3	3·4	1·8	1·2	1·9	1·7	1·3
Leucine	6·3	6·5	4·0	2·5	2·9	2·2	1·8
Tyrosine	2·0	1·9	6·8‡	13·9‡	10·4‡	13·2‡	8·8‡
Phenylalanine	2·4	2·2					
Basic	12·8	12·5	23·0	9·1	13·9	8·6	14·8
Acidic	28·1	27·8	19·0	16·8	15·1	13·5	14·0
Neutral	59·1	59·7	58·0	74·1	71·0	77·9	71·2
Total (μmoles/100 mg DNA)	4·653	4·750	8·196	2·773	2·147	4·195	6·467

* Each sample contained pooled tissues from fourteen male CFE rats. Details of the dose regimen are given in Experimental, and those of DNA isolation and amino acid analysis, in earlier studies.[29, 30] Abbreviations used include: NT, no treatment; HN2, nitrogen mustard; Act D, actinomycin D. Unless otherwise specified, values are expressed as mole per cent, calculated from the total amino acids determined. The plus sign (+) designates presence in trace amounts.

† Neither methylvaline nor sarcosine was present in these hydrolysates.

‡ Values represent a single unresolved aromatic peak, calculated by the absorbance method, using the average of phenylalanine and tyrosine as the constant.[30]

There were still further variations which were specific for the particular compound or regimen of treatment. Due to the increase of all three basic amino acids in HN_2-DNA, the mole fraction of this group was about twice that of the controls. The total neutrals remained unchanged in spite of changes in almost all of the amino acids. After treatment with antibiotics, lysine and histidine decreased, resulting in a decrease in total basics, except for the multiple-dose sample, where the increase in arginine more than compensated for the decrease. In actinomycin D-DNA, there was an increase in threonine in addition to all those mentioned above. Concomitant with the loss of acidics in all these samples, there was a large increase in neutrals. Increases in histidine and threonine along with the other general changes resulted in almost equal amounts of acidics and basics in proflavin-DNA.

DISCUSSION

Earlier studies have shown that the amount of amino acids bound to bacterial DNA was a function of growth, varying not only with changes in the composition of the medium but also with the phase of the growth cycle.[31, 33] Moreover, when cultures were transferred from enriched to minimal medium, or vice versa, the changes in the total amounts of these residues occurred very rapidly.[36] Similarly, physiologic changes

can be elicited in whole animals by partial hepatectomy, chemical induction of tumors, or treatment with drugs. Both regenerating liver and induced hepatoma showed increases in total residues and changes in composition when compared to normal liver.[30]

Among the amino acid residues, there are presumably some which are actually attached to DNA and others which are components of specific peptides appended to them. The latter amino acids, which constitute the majority of those present, could no doubt be readily modified by physiologic changes and account for the qualitative and quantitative variations observed. The functions of these two kinds of amino acids are also probably not identical.

The data presented here indicate that the amount and nature of the amino acids bound to the DNA of a particular tissue can indeed be modified by physiologic changes following drug treatment. Although the two dose regimens of mitomycin C did not appreciably affect the relative distribution of the amino acids, the single dose treatment had a more pronounced effect on the total residues of all tissues except liver. Tomisek *et al.*[37] have observed differences in enzymatic activities of tumors after single and multidose treatment with cyclophosphamide. The variations in the experimental parameters in DNA from a particular tissue were not unexpected in view of the differences in the biochemical effects of the four compounds studied. It seems rather unlikely that the changes were merely due to nonspecific impairment of DNA synthesis resulting from general toxicity and debilitation of the animals, since rather pronounced changes were observed within 1 hr after a single dose of mitomycin C.

The apparent lack of a consistent pattern in the changes of the amino acids bound to DNA of the various tissues after treatment with a particular compound emphasizes the complex nature of the response of each tissue. The total amounts may be increased in one tissue and yet be markedly reduced in another, e.g. after mitomycin C treatment. Even when the total residues were increased in all the tissues examined, there appeared to be considerable variations in the relative distribution of the amino acids, e.g. after HN2 treatment. The control tissues exhibited the characteristic tissue variations previously reported.[29] The changes observed after therapy no doubt represent the end result of the interplay between such inherent tissue variations and those elicited by the response of the particular tissue to the drug. Since all four compounds interfere with some phase of the synthesis of nucleic acid or protein, or of both, found in all cells, it is conceivable that the differences in tissue response are manifestations of the inhibition of certain functions which are characteristic of the particular tissue. Unfortunately, no simple correlation could be found between the changes in the amino acids bound to DNA and the type of inhibition caused by a compound. Actinomycin D and proflavin, for example, are both known to interfere indirectly with protein synthesis.[4, 11, 16] Yet, in liver, a major protein-synthesizing tissue, treatment resulted in a striking increase of total residues with actinomycin D and a small but definite decrease with proflavin. The relative amounts of the three groups of amino acids were not too different, although minor variations existed within each of these groups. It would be tempting to suggest on the basis of these data that the relative distribution of the three groups of amino acids is a consequence of the inhibition of certain cellular functions, whereas the quantitative changes in total residues are the characteristics of the particular inhibitor–DNA interaction. The results obtained with small intestines, a rapidly proliferating tissue, after treatment with compounds capable

of blocking cell replication,[8, 15] argue against such a suggestion. Therapy resulted in an increase in total residues with HN2 and a decrease with mitomycin C. However, unlike the DNA samples discussed above, there are considerable variations in the relative distribution of most of the amino acids.

The variations in the individual amino acids are too complex to assess at the present time. Nevertheless, in small intestines, a rapidly dividing tissue, a number of qualitative changes appear to be common with all four compounds, namely, decrease in both aspartic and glutamic acid, increase in serine, and increase in aromatic amino acids. The latter not only increased in relative amounts, but also appeared as a single unresolvable peak. Similar peaks associated with isolated DNA have previously been reported in bacteria during early growth phases,[31] in some spontaneous primary tumors,[30] in some chemically induced tumors and their initial transplants,[30, 34] and even in transplanted tumors such as S180 after treatment with 1,3-bis(2-chloroethyl)-nitrosourea.* This extra component has not been found in the hydrolysates of total cellular proteins.[30, 34] Preliminary experiments indicate that there is at least one extra ninhydrin-positive component in this peak, which behaves chromatographically like the aromatic amino acids in the Moore–Stein system,[38] but not in simple paper chromatography with solvents such as *n*-butanol–glacial acetic acid.† The identity and possible metabolic implications of this component are subjects of current investigation.

Although the addition of proflavin *in vitro* appeared to modify the gross physical characteristics of liver DNA, no apparent changes could be observed in either the amounts or the nature of the amino acid residues. The absence of proflavin in the purified DNA is consistent with the reversibility of such aminoacridine–DNA complexes.[11, 16] Consequently, it is conceivable that the decreases in acidics, basics and total residues as well as the increases in a number of the neutrals when proflavin was present *in vivo* are reflections of actual metabolic changes pursuant to treatment and not merely the result of indiscriminate associations of cellular proteins concomitant with intercalation of proflavin in DNA. The lack of radioactivity in DNA when labeled amino acid was present in the homogenate indicates that there was no random adsorption of individual amino acids on the DNA during the isolation process.

Superficially, the increase in total residues after treatment with HN2 is reminiscent of the well documented increase of proteins cross-linked to DNA.[22, 25–27] However, a closer examination of the relative distribution of the amino acids indicates that, in contrast to the increase in dicarboxylic acids found in such cross-linked proteins,[39] there was actually a decrease in acidics and a simultaneous increase in basics in all tissues except spleen. Despite the decidedly basic character, there appears to be no apparent similarity in composition to the nuclear histones. Furthermore, random cross-linking of cellular proteins by HN2 seems rather unlikely from four different lines of experimental evidence. First, the values for liver DNA after HN2 treatment were the average of two independent isolations, which were reproducible within 3 per cent of each other. It is difficult to reconcile such close replication with a purely random process. Secondly, the disproportionate increase in arginine in liver DNA would require the linking of an extraordinary protein with about 50 per cent arginine. Thirdly, after treatment, there were losses in the absolute amounts of some of the

* J. S. Salser and M. E. Balis, unpublished observations.
† J. S. Salser and M. E. Balis, unpublished observations.

amino acids present in the control tissue. Fourth, the extra component found in DNA from small intestines has not been detected in the hydrolysates of the residual proteins of any such DNA samples.[31] These data suggest that the amino acids bound to DNA are entities which are distinct and separate from the cellular proteins, some of which can presumably be linked by HN2, but are lost during the subsequent purification procedure. The relatively large increases in the total residues of some tissues after treatment with actinomycin D and proflavin, neither of which is capable of such cross-linking, further argue against simple cross-linking of cellular proteins to DNA by the agent as an explanation for the increase in amino acids associated with DNA.

The entrapment of proteins as a result of cross-linked DNA strands also seems rather unlikely, since treatment with mitomycin C, a compound known to form stable DNA complexes of this type,[8] did not show any spectacular increases in total residues when compared to HN2 and, in small intestines, actually showed a marked decrease. Since mitomycin C can also act as a monofunctional alkylating agent, the decrease in total residues emphasizes that alkylation plays a negligible role, if any, in the quantitative changes of the amino acids. The possibility of contribution from cyclic peptides from bound actinomycin D has been ruled out by virtue of the reversibility of such DNA complexes[4] and by the absence of the unusual amino acids, sarcosine and methyl valine, in the DNA hydrolysates.

In conclusion, treatment with compounds which react with DNA, be it by covalent linkages or strong coordinate or π-bond complexes, does result in quantitative as well as qualitative changes in the amino acid residues associated with DNA. Such changes appear to require host interaction, as was exemplified by the absence of change when proflavin was added *in vitro*. Although it is impossible to correlate any particular pattern of the experimental parameters with specific changes in DNA function, the data indicate that the quantitative changes can not be merely attributed to simple addition of peptides to those inherent in the tissue or alternatively to simple subtraction from these innate residues. These changes occurred with relatively little change in the distribution of the three groups of amino acids in some tissues and with rather striking changes in others. There were decreases in the absolute amounts of certain amino acids concomitant with increases in total residues, e.g. lysine in liver DNA with mitomycin C, histidine in spleen DNA with actinomycin D, aspartic acid in liver DNA with HN2. Conversely, there were increases in individual amino acids concurrent with a decrease in total residues, e.g. alanine in liver DNA with proflavin, aromatic amino acids (no doubt due to contributions of the extra component) in DNA from small intestines with mitomycin C. These data would tend to suggest that there were also substitutions of new amino acid residues, probably in the form of peptides, for those initially presentin the tissue. Moreover, these new "peptides" appear to be different from the bulk cellular proteins. At least one extra component has been found in the residues bound to DNA, but not in the corresponding cellular proteins. Similarly, the exceptionally high arginine-containing component in liver DNA after HN2 treatment has no known counterpart among liver cell proteins. An analogous situation has been reported in studies with bacteria growing under conditions of thymineless death, where radioactivity was incorporated into total cellular proteins but not into the residues bound to DNA.[31]

Recent studies with a number of alkylating agents indicate that there was a time delay between the administration of a compound and the appearance of detectable

inhibitory effects.[15] Moreover, there was a transient stimulation of growth and metabolic activities,[15, 37, 40] which was attributable to the systemic effects of the compounds upon the animals. Stimulations of this nature would not only account for the increases observed in total amino acid residues bound to DNA after treatment with alkylating agents, but would also be consistent with an involvement of these residues in cellular functions in a manner reminiscent of bacterial systems.[31, 33] The changes resulting from the final dose could be large enough to mask most of the effects of the chronic doses. Alternatively, they could be too small to modify appreciably the cumulative effects of such chronic doses; this could result in an actual decrease in total residues. The net result of changes such as these could help to explain the differences observed with the two different dose regimens of mitomycin C. Although there are no data available at present, it is conceivable that analogous systemic effects would result from treatment with the other compounds.

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