RADIOACTIVE TRACER STUDIES ON ARSENIC INJECTED AS POTASSIUM ARSENITE

II. CHEMICAL DISTRIBUTION IN TISSUES

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The preceding paper has shown that small amounts of arsenite tagged with As^{74}, when injected into the body, become distributed in the various tissues at concentrations more or less characteristic for each tissue. These arsenic concentrations, in general, are much greater in the tissues than in the serum. This suggested that a non-diffusible combination of the injected arsenic is formed within the cells. The present paper presents the results of our investigations of the manner in which arsenic is held by the tissues after its administration.

Because of its extreme sensitivity the radioactive tracer method has proved of great advantage in this work. For example, following the administration of a non-toxic amount of arsenite containing As^{74}, a sample of liver had so much radioactivity that only 3 mgm. of tissue, or an equivalent fraction of a larger tissue sample, were required for analysis. This amount of tissue contained only 10^{-6} mgm. of arsenic.

Voegtlin, Dyer and Leonard (6) suggested that the sulfhydryl groups in reduced glutathione and in proteins combine with trivalent arsenic, and Rosenthal (7) observed the *in vitro* combination of arsenoxide (*3*-amino-*4*-hydroxyphenyl arsenious oxide) with proteins in parallel with their content of free sulfhydryl groups. However, it was not possible for them to demonstrate that arsenic combined exclusively with sulfhydryl groups. The further possibility exists that arsenic might replace phosphorus, since arsenate will apparently substitute for phosphate in at least one step in the glycolytic cycle, and it has been reported that liver nucleoproteins will take up arsenic (8). To investigate the relationship of arsenic to phosphorus was one object of our work.

The first step in tracing the arsenic was to divide the tissues into acid-soluble, lipid, and protein fractions. The acid-soluble fraction should contain (*a*) inorganic arsenic, (*b*) arsenic which might have replaced phosphorus in the acid-soluble organic phosphorus compounds, and (*c*) any arsenic in combination with reduced glutathione. Following this general fractionation, the protein portion, which was found to contain the bulk of the arsenic, was further fractionated, chiefly with ammonium sulfate. Analyses for phosphorus as well as for arsenic were made on the various fractions to see if any correlation could be observed in the distribution of these two elements.

Although the arsenic did not prove to be confined exclusively to any group of proteins, definite differences were observed in the amount of arsenic in different protein fractions.

METHODS. The tissue acid-extracts were prepared with 0.75 M nitric acid. The lipids were extracted from the acid-insoluble residue with alcohol and ether. The lipid-free

residue was taken as the "protein" fraction although it obviously would contain some glycogen. These fractions were then digested with sulfuric and fuming nitric acids, a definite volume of 3% sodium bicarbonate was added to each sample, and aliquots were taken for measuring the radioactivity and phosphate (9).

For the protein fractionation, the tissue was minced and then thoroughly broken up in a Waring blender with ice cold 1% sodium chloride solution at pH 7.4. The portion of the tissue in solution after this treatment was used for fractionation of the proteins with different concentrations of ammonium sulfate (6°C.).

In order to precipitate each fraction a number of times with ammonium sulfate without too much loss, a scheme of precipitation was used which was analogous to the procedure employed in making repeated fractional distillations. After making the initial successive precipitations (*1a*, *1b*, etc.), the fraction *1a*, insoluble in the lowest salt concentration (0.6 M $(NH_4)_2SO_4$), was dissolved insofar as possible in water, centrifuged, and precipitate *2a* obtained by adding to the separated supernatant fluid a sufficient quantity of ammonium sulfate to bring the salt concentration to 0.6 M once more. At this point instead of discarding the resultant supernatant, it was used to redissolve precipitate *1b*, previously obtained, with the next higher salt concentration (1.2 M). Any of this precipitate which failed to redissolve in the 0.6 M ammonium sulfate was combined with precipitate *2a* for succeeding reprecipitations. Meanwhile precipitate *2b* was obtained by adding ammonium sulfate to the supernatant liquid to obtain a concentration of 1.2 M. This general procedure was followed with each of the other fractions obtained with increasing ammonium sulfate concentrations. By thus retaining all of the supernatant fluids, the only protein lost was that which failed to dissolve in water at one end of the scale, and that which failed to precipitate with the highest ammonium sulfate concentration (3.6 M) at the other. Each fraction was precipitated four times, following which it was washed with 10% trichloracetic acid, then with a 1:3 alcohol-ether mixture, dried, weighed, wet-ashed, and finally analyzed for phosphorus and arsenic.

In addition several samples of nucleoprotein were prepared. The method of Greenstein (10) was followed initially, but since this involves bringing the preparation to pH 10.5, and since at this pH arsenic was found to be partially split from the proteins, the procedure was modified to avoid pH's above 8.5. As a result, the yield was decreased, but the phosphorus content was increased from about 1% to over 4%.

RESULTS. In Table 1 is recorded the distribution of arsenic and phosphorus in the acid-soluble, lipid, and "protein" fractions obtained from five tissues and the serum of a guinea pig, and from the liver and kidney of a chimpanzee and of a baboon. The guinea pig had been injected 8 times with arsenite over a period of 4 days, the chimpanzee had received a single injection 24 hours previously, while the baboon had been injected one week before the samples were obtained. With the exception of the serum, the protein fraction appeared to contain the bulk of the arsenic, with a variable amount in the acid-soluble fraction and a negligible amount in the lipid fraction.

Not all of the acid-soluble arsenic was dialyzable through a cellophane membrane. It may be mentioned at this point that in contrast to the findings in regard to the tissues, in urine, serum, and bile practically all of the arsenic was found to dialyze readily through a cellophane membrane.

In the case of liver and kidney, the tissue proteins were further fractionated to see if the arsenic was concentrated in any particular group. Some typical data for the liver of the same chimpanzee mentioned above are shown in Table 2. Per gram of protein there was three times as much arsenic in the less soluble pro-

TABLE 1

Distribution of arsenic and phosphorus in fractions of various tissues (values reported per kg. of fresh tissue)

	ACID-SOLUBLE			LIPID			PROTEIN		
	As 10^{-7} mol	P 10^{-3} mol	As/P x10^4	As 10^{-7} mol	P 10^{-3} mol	As/P x10^4	As 10^{-7} mol	P 10^{-2} mol	As/P x10^4
Guinea pig									
Liver	3.5	36.1	.097	.5	37.0	.01	32.6	31.4	1.04
Kidney	2.7	26.2	.103	.7	26.6	.03	29.0	28.4	1.02
Spleen	<.3	39.5	<.01	<.3	17.5	<.02	8.4	50.8	.14
Brain	.4	24.9	.016	.1	59.7	.002	11.7	15.5	.75
Muscle	.2	59.4	.004	.2	10.9	.02	9.1	4.8	1.90
Serum	.8	.70	1.1	.1	.32	.3	.06	.06	1.0
Chimpanzee #112									
Liver	4.0	32.6	.12	.1	24.8	.006	10.6	25.3	.42
Kidney	3.7	38.3	.10	.1	23.0	.004	5.9	15.1	.39
Baboon									
Liver	.9	34.0	.03	.4			5.7	23.4	.24
Kidney	1.9						8.1	26.8	.30

TABLE 2

Distribution of arsenic and phosphorus in protein fractions of the liver

PROTEIN FRACTION	PER CENT OF TOTAL PROTEIN	As 10^{-7} MOLS PER KG. PROTEIN	P 10^{-3} MOLS PER KG. PROTEIN	As/P x10^4
Chimpanzee #112				
Whole liver	100	56	133	.42
Sol. in 1% NaCl	82	60		
Sol. 1% NaCl; ins. H_2O	4	76	152	.50
Sol. H_2O; ins. .6 M*	13	69	163	.42
Sol. .6 M; ins. 1.2 M*	15	61	226	.27
Sol. 1.2 M; ins. 1.8 M*	19	43	61	.70
Sol. 1.8 M; ins. 2.7 M*	17	28	62	.45
Sol. 2.7 M; ins. 3.6 M*	2	23	61	.38
Sol. 3.6 M; ins. 10% CCl_3COOH†	4	27	40	.67
Baboon				
Whole liver	100	30	123	.24
Nucleoprotein	.7	28	1350	.02

* Molarity of ammonium sulfate.

† Precipitate with trichloracetic acid from the last supernatant removed from previous fraction.

tein fractions than in the more soluble fractions. Similar results were observed for other liver samples and a kidney specimen. Although a relationship seemed to exist between solubility and arsenic content, the proteins initially insoluble in 1% saline were not found to be any higher in arsenic than those fractions initially soluble in the dilute sodium chloride solution. The tendency for the values for arsenic and phosphorus to parallel each other in the liver (table 2) was not found when kidney tissue was investigated, for in that tissue the highest phosphorus values were obtained in the most soluble fractions.

In regard to nucleoprotein, the arsenic concentration did not appear to be any higher than the average obtained for other proteins (see Table 2).

TABLE 3

Removal of arsenic and phosphorus from liver proteins under various conditions

TREATMENT	As PER CENT INITIAL VALUE	P PER CENT INITIAL VALUE
Chimpanzee #86		
Rapid isolation with 10% CCl_3COOH	100	100
10% CCl_3COOH 18 hours 25°C	105	32
N/10 HCl ¼ hour 100°C	48	70
N/10 HCl 1 hour 100°C	33	47
4% Borax 1 hour 25°C	55	80
N/10 NaOH 1 hour 25°C	4	98
Baboon		
Rapid isolation with 10% CCl_3COOH	100	
pH 7.4 1 hour 25°C	97	100
pH 8.0 1 hour 25°C	91	
pH 8.5 1 hour 25°C	85	
pH 9.0 1 hour 25°C	75	101
pH 9.7 1 hour 25°C	60	
pH 12.5 1 hour 25°C	35	87
N/10 HCl 0.16 hour 100°C	56	56
N/10 HCl 0.5 hour 100°C	41	47
N/10 HCl 1.0 hour 100°C	38	34
N/10 HCl 2.0 hours 100°C	35	34

Evidence against the replacement of phosphorus in proteins by arsenic may be found in the difference in the behavior of the two elements on hydrolysis (Table 3). In contrast to phosphorus, arsenic appears to be very susceptible to the action of cold alkali, and insensitive to cold acid, a behavior compatible with a combination with sulfhydryl groups. On the other hand, both arsenic and phosphorus show a similar behavior toward hot acid, for after a decrease to about one-third the original value a limit appears to be reached. This suggests the presence of two types of bonds for both elements in their combination with protein.

SUMMARY

1. Following the administration of arsenite tagged with As^{74}, the distribution of arsenic in various fractions of mammalian tissues has been investigated.

2. The bulk of the arsenic was found in the protein fraction, with a much smaller amount in the acid-soluble portion and only an insignificant amount in the lipid fraction.

3. The arsenic was not evenly distributed throughout the proteins, but in general appeared to be more concentrated in those tissue proteins which are precipitated by less than half-saturated ammonium sulfate.

4. The nucleoproteins did not take up more arsenic than proteins in general.

5. From the comparative distribution and behavior on hydrolysis of phosphorus and arsenic, there is little evidence for the replacement of phosphorus by arsenic in the tissues.

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