AN ATTEMPT TO ASSESS BLOOD GROUPS IN PALEOANTHROPOLOGICAL MATERIAL

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The successful assessment of blood groups in forensic medicine (e. g. in dried blood stains) stimulated the idea of using a similar serological method for assessing blood group substances in paleoanthropological material. So far there are few publications in international literature on paleoserology. The interest in paleoserology is divided into assessment of group substances in mummified tissue (2, 3, 4, 5, 6, 16, 17, 18, 21, 22, 34) and assessment of group substance in osseous tissue (8, 9, 10, 11, 12, 13, 19, 25). While the results of serological examinations of tissues from mummies seem to be more successful, the results obtained with bone material are not quite satisfactory and still many obscure problems remain open.

We therefore welcomed the opportunity of carrying out paleoserological examinations on a group of human skeletons from the archeological site in Mikulčice, Moravia. The skeletons from this important Moravian centre, which has been investigated systematically by the Archaeological Institute of the Czechoslovak Academy of Sciences in Brno under the guidance of J. Poulík at the "Valy" site near Mikulčice for several years, is important in many respects. First of all it is one of the largest of its kind and it provides a reliable basis not only for recognising the somatic characteristics of old Slavs but also for investigating a number of other research problems. The skeletons in Mikulčice were discovered on at least five independent burial sites, completely and more or less remotely separated, and the findings from these five sites can be mutually compared. The question arises of what reasons caused five cemeteries in ninth-century Mikulčice to be used simultaneously. Different factors may have been involved — social, religious, family, etc. The possibility cannot be ruled out that the separation of the population was connected in some way with the fact that apart from the local component there were also influences from abroad, particularly relations with the south-east and perhaps also the west and north, which were also reflected to a varying extent in the biological structure of the population.

Every burial place has its peculiar features by which it differs from the rest. The third burial place (graves the numbers of which are divided by IV) are particularly striking in this respect. It is relatively small and it seems to be the "private" burial place of a family. It differs from the other Slav burial places by its demographic structure, most of the buried people being women. It cannot be ruled out that this fact suggests, considering other circumstances, the persistence of polygamy in the ruling family of Mikulčice. But also on analysis of further characteristics the third burial place differs from the others, e. g. from the first one, from which the second sample used in the present work was taken.

The archeological site of "Valy" is situated in the inundation area of the Morava river. The soil is mostly sandy, rich in limestone. Floods from above, however, apparently did not affect the skeletons directly as over most of the graves there was a layer of intact soil. It cannot be, however, ruled out that the skeletons were exposed to the action of ground waters.

MATERIAL AND METHODS

A total of 63 skeletons from grave IV and 61 skeletons from grave I were processed (*tables 1*

TABLE 1
List of skeletons from the third burial place in Mikulčice

Specimen	Sex	Age	Specimen	Sex	Age
1/IV	?	juvenis	48/IV	woman	maturus
2/IV	woman	maturus	50/IV	woman	?
3/IV	?	infans II	51/IV	man	maturus
4/IV	man	adultus	53/IV	woman	adultus
5/IV	woman	maturus	61/IV	?	juvenis
6/IV	?	infans I	62/IV	woman	maturus
8/IV	?	infans I	63/IV	woman	adultus
9/IV	woman	adultus	64/IV	?	infans II
10/IV	?	infans II	66/IV	woman	adultus
12/IV	?	juvenis	68/IV	man	maturus
13/IV	man	maturus	69/IV	man	maturus
14/IV	man	maturus	71/IV	man	maturus
15/IV	woman	?	74/IV	man	maturus
17/IV	man	adultus	75/IV	woman	adultus
18/IV	man	adultus	76/IV	man?	juv. —
19/IV	man	maturus			adultus
20/IV	man	maturus	77/IV	man	maturus
21/IV	woman	adultus	79/IV	man	maturus
22/IV	woman	maturus	80/IV	man	adultus
27/IV	man	maturus	81/IV	?	infans II
28/IV	?	infans I	82/IV	woman	maturus
29/IV	man	maturus	85/IV	woman	maturus
31/IV	woman	maturus	86/IV	man	maturus
33/IV	?	infans II —	88/IV	?	infans II
		juv.	89/IV	?	infans II
34/IV	woman	maturus	90/IV	woman	adultus
39/IV	?	juvenis	92/IV	woman	adultus
40/IV	man	maturus	94/IV	?	infans I
41/IV	?	infans I	95/IV	woman	adultus
42/IV	woman	adultus	97/IV	woman	adultus
44/IV	woman	maturus	99/IV	?	infans I
45/IV	woman	?	100/IV	?	?
46/IV	woman	adultus			

and *2* contain more detailed data on the sex and approximate age). For testing group substances flat bones were used (ribs, pelvic bones) or the epiphyses of long bones. In particular those bones were processed which were preserved *IN TOTO*. The ske-

TABLE 2
List of skeletons from the first burial place in Mikulčice

Specimen	Sex	Age	Specimen	Sex	Age
1/I	woman	juvenis	78/I	?	infans II
6/I	woman	adultus	80/I	woman	adultus
10/I	woman?	infans I	82/I	man	maturus
13/I	man	maturus	83/I	man	maturus
20/I	woman	adultus	84/I	man	maturus
21/I	woman	adultus	90/I	man	senilis
26/I	woman	adultus	92/I	woman	maturus
28/I	man	maturus	94/I	man	maturus
30/I	man	adultus	96/I	man	maturus
34/I	man	maturus	97/I	woman	maturus
35/I	woman	adultus	98/I	woman?	infans I
36/I	woman	adultus	99/I	woman	maturus
37/I	man	adultus	102/I	man	maturus
39/I	man	maturus	114/I	woman	maturus
42/I	man	maturus	116/I	man	adultus
43/I	man?	maturus	117/I	man	maturus
44/I	man	maturus	119/I	man	maturus
46/I	?	infans I	120/I	man	senilis
51/I	woman	senilis	122/I	man	senilis
52/I	woman?	adultus	123/I	?	infans I
54/I	?	infans I	124/I	?	infans I
56/I	woman	maturus	126/I	woman	maturus
58/I	woman	maturus	131/I	woman	maturus
59/I	man	maturus	132/I	woman	maturus
65/I	woman?	juvenis	134/I	?	juvenis
66/I	man	senilis	137/I	man	maturus
68/I	?	infans II	143/I	man?	juvenis
73/I	?	infans I	157/I	woman	adultus
74/I	?	infans I	159/I	?	infans II
76/I	woman?	infans I	162/I	man?	juvenis
77/I	?	infans I			

letal remnants were mechanically cleaned, washed, dried and stored in cardboard boxes in a dry place. Between their discovery and the processing in the laboratory several months elapsed. The best preserved parts of the skeletons were selected, put into bags, marked and sent to the laboratory for serological processing. Only those bones were examined which were not preserved by lacquers or other methods.

For the serological examinations only cancellous was used and not the cortical layer. The bones were broken and the internal cancellous material was scraped out mechanically with a steel knife. Thus coarsely crushed bone was obtained which was ground in porcelain mortars to a powder. The latter was kept in test tubes closed with a cork stopper and processed at the earliest opportune moment, i. e. within several days.

For testing group substances in this material, absorption tests were used. These were carried out in several modifications; however, most experiments were carried out as follows: (Method A) 0.7 g of bone powder was weighed and combined with 0.6 ml of diagnostic serum, the mixture was left at room temperature or in a refrigerator for three hours. After this period the supernatant was removed by centrifuging (2500 rotations/min. for three minutes) and used for titration. The titration was made against blood cells of group A_1 and B in agglutination test tubes by gradual double dilution of the diagnostic serum (as a control) and supernatant with physiological saline; to every test tube the same volume (0.1 ml) of a 2 % suspension of red blood cells in physiological saline was added; after standing for 15 mins. at room temperature the test tubes were centrifuged at 1000 rotations/minute for 2 minutes.

(Method B) Into the test tubes provided with a mark at the same volume, the crushed bone was added up to the mark and wetted with the same volume of physiological saline; after several minutes the sample was centrifuged (2500 rotations/minute for three minutes), the physiological saline was removed from the sediment and the same amount of diagnostic anti-A serum as of the sediment was added; the same procedure was used with anti-B serum. After shaking and mixing the suspension was left for three hours at room temperature. Then the mixture was centrifuged and the supernatant treated in the same way as in method A.

Selection of diagnostic sera: in the first experiments, which are not described in the present paper, diagnostic anti-A and anti-B sera were used, the potency of which was adjusted only by dilution with physiological saline. In view of the possible objection that more diluted serum contains less protein than less diluted serum of the same antibody potency, and that this fact may play a role in absorption tests (non-specific adsorption of antibody protein in slightly diluted serum will be less marked than in greatly diluted serum) serum donors were selected who had practically equal antibody titres, i. e. donors of group B for anti-A with a titre of 1 : 64 and donors of group A for anti-B with the same titre, and thus it was not necessary to adjust the potency of sera by a different dilution with physiological saline.

Apart from absorption tests with sera anti-A and anti-B a number of experiments were made with anti-Rh sera. Saline agglutinating anti-D, anti-C and anti-E sera were used, the titration results of which are listed in *table 11.*

Absorption: to crushed bone, physiological saline was added and left in the test tubes in the refrigerator at 4 °C overnight (18 hours). On the next day the mixture was centrifuged, the physiological saline was poured off and the wetted crushed bone was rinsed once with sterile physiological saline. After centrifuging and removal of physiological saline, to the crushed bone 0.1 ml antiserum was added. The amount of dry bone used in all tests was such as to fill the agglutination test tubes to the same volume as 0.1 ml of fluid. The mixture was incubated for three hours in a thermostat at 37 °C.

The supernatant was sucked off after centrifuging and titrated by consecutive double dilution with physiological saline in microtest tubes in the usual

manner, using a marked Pasteur pipette. Then the corresponding type of blood cells in a 2 % suspension of physiological saline were added. The incubation period was one hour at 37 °C. The reaction was evaluated by microscopic examination. The results were compared with control titrations of antisera; in these titrations the slight dilution of antisera with physiological saline which even after sucking off may remain in the sediment was neglected.

Estimation of dissolved substances (method C): In order to decide whether after leaving the crushed bone in physiological saline for a prolonged period group active substances leach into the solution, the following experiment was carried out: to the crushed bone the same volume of physiological saline was added and left to stand for 48 hours at 4 °C. After centrifuging the physiological saline was removed and mixed at a ratio of 1 : 1 with antiserum (anti-A and anti-B) and the mixture left for three hours at room temperature and processed subsequently, as decribed above for the absorption of anti-A and anti-B antisera. — The remaining crushed bone from this experiment was tested for the content of substances: after removing the physiological saline antiserum was added to the sediment: 0.7 g of the original dry crushed bone and 0.6 ml antiserum. The mixture was left for three hours at room temperature and the supernatant was titrated, as described above, for absorption experiments with anti-A and anti-B sera.

Repeated absorption with the same bone material (method D): the crushed bone was used in absorption test for three hours at room temperature and then the antiserum was removed and titrated; to the remaining crushed bone new antiserum was added and the mixture was allowed to stand overnight till the next day, i. e. for 18 hours in a refrigerator at 4 °C. Then the antiserum was sucked off after centrifuging and another titration was made. To the bone sediment again antiserum was added and was left again for 48 hours in the refrigerator. Then after centrifuging the supernatant was removed and titrated.

A similar experiment in a different modification (method E): The same bone material was repeatedly (four times) used for absorption tests on consecutive days. To 0.7 g of crushed bone 0.5 ml of the appropriate antiserum was added, left for three hours at room temperature, centrifuged and the supernatant was titrated; on the same day to the crushed bone new antiserum was added, left to the next day at room temperature, centrifuged and titrated; in the same way the same tests were carried out on the following two days.

Experiment with repeated absorption of the given antisera with homologous bone material (method F): The crushed bone was left overnight, combined with physiological saline which was sucked off the next day and the sediment was combined with diagnostic serum at a ratio of 0.7 g crushed bone per 0.5 ml serum. The absorption was carried out for three hours at room temperature. The same was carried out in an independent experiment on the next day. The sediments (homologous) from both experiments were not used but after absorption the sera were combined on the next day and used for the absorption test with the new homologous crushed bone: the absorption was carried out for three hours at room temperature at a ratio of 0.5 g to 0.3 ml serum. Then after centrifuging the supernatant fluids were titrated.

Check-up examinations of the soil specimens from the area where the skeletons were found: As the possibility cannot be ruled out that the substance in the absorption tests causing the decline of the isoagglutinin titres may not originate from the bones but may have been acquired secondarily from the surroundings, e. g. the soil, experiments were conducted with the soil where the skeletons or bones were found. At the same time soil specimens were collected which were not directly connected with the skeletal remnant. A total of four soil specimens were taken and processed in absorption tests: 1. underlying soil (niveaux) in direct contact with the skeleton, 2. another underlying soil specimen in direct contact with the skeleton, 3. find-stratum, 4. a specimen from a layer 40 cm underneath the underlying soil. To every specimen (2 g) 1.5 ml diagnostic serum were added. The absorption was made for three hours at room temperature. The sample was briskly centrifuged, the supernatant was removed and examined serologically. The experiments were carried out three times independently.

Control experiments with some salts contained in osseous tissue: these experiments were to answer the question of to what extend the results of serological experiments may be influenced by the chemical composition of bone, whether the salts forming bony tissue can influence in an adverse manner the results of absorption tests (either directly by their adsorption ability or by exerting an influence on the serological reaction). We made absorption experiments with the following compounds: 1. calcium phosphate, 2. magnesium phosphate, 3. calcium carbonate. The methods were the same as those used for testing the soil specimens. Three independent experiments were carried out.

Chemical analysis of crushed bone. Crushed bone from skeletons marked 4/IV, 5/IV, 9/IV, 12/IV, 14/IV, 18/IV, 20/IV, 22/IV, 27/IV, 29/IV, 31/IV, 33/IV, 34/IV, 79/IV, 82/IV, 95/IV, 97/IV, 100/IV were used for testing which chemical substances related to group substances A and B were preserved up to the present time. In a control trial also soil (specimen 1) was processed. Several preliminary trials were made (1—3) to assess amino acid, sugars and proteins and other experiments (4 and 5) to identify individual components more closely.

Experiment 1: 15 g crushed bone were eluated four times with 25 ml butanol, saturated with H_2O for 20 hours. Individual eluates were combined and evaporated on a water bath *IN VACUO* to a volume of 0.5 ml. This extract was transferred to chromatographic Whatman 3 paper and separated at a potential gradient 9.3 V/cm in a pyridine-acetic acid-water buffer (10 : 4 : 450) pH 5.3, for two and a half hours. Then the electrophoreogram was well dried

and for detection a 0.1 % solution of ninhydrin in acetone was used.

Experiment 2: 10 g of crushed bone were eluated six times with 10 ml distilled water for 20 hours. The eluates were combined and evaporated to a volume of 10 ml. In 5 ml the sugar content was estimated using a combination of Somogyi's (29) and Nelson's (24) method, compared in a blank experiment and with a standard glucose solution (10, 50 and 100 gamma). Another 5 ml of eluate were heated with 1.5 ml 1 N HCl for 45 minutes at 100 °C and reducing sugars were estimated, similarly as described above.

Experiment 3: 10 g of crushed bone were eluated with physiological saline for 5 hours. Then the bone material was filtered off and 5 ml of the filtrate were used for estimating proteins by means of the phenol reagent (modification of Folin's method) (20).

Experiment 4: 50 g of crushed bone were eluated six times with 50 ml distilled water for 20 hours. Individual eluates were combined to a volume of 300 ml. The combined eluates were subsequently evaporated to a volume of 10 ml. From this a 2.5 ml portion was taken and evaporated to 0.25 ml; this amount was transferred to Whatman 3 chromatographic paper and separated electrophoretically under the same conditions as in experiment 1. After drying the electrophoreogram, standards of most common amino acids were applied to its margin. To the longer side of the electrophoreogram another chromatographic paper was fixed and descendent chromatography in a perpendicular direction to the electrophoretic separation was made. As a developing system the following solutions were used: n-butanol-acetic acid-water (4 : 1 : 5); the chromatogram was developed for "overflow", as indicator of the course of the most rapid amino acid — leucine, tropaeolin was used. The chromatogram was dried at 95 °C and for detection a 0.1 % solution of ninhydrin in acetone was used. The evaluation was made by comparing the chromatographic spots of the bone material with standards (for method see experiment 3).

To the remaining concentrated eluate, 3 ml 96 % alcohol with 0.5 ml Na_2SO_4 were added and the mixture was left overnight in the refrigerator at 5 °C. The precipitate formed was centrifuged. Half the precipitate was transferred into a narrow tube and dissolved in a small amount of distilled water. To this solution sufficient concentrated HCl was added to obtain a final concentration of 6 N HCl. The tube was sealed and left for six hours at 100 °C. Then it was opened, the contents were evaporated to dryness, and the evaporate dissolved in 0.2 ml distilled water. The hydrolysate thus obtained was further separated electrophoretically and subjected to chromatographic examination under equal conditions as in the first part of the experiment. The second portion of the precipitate was used for estimating bound or non-reducing sugars. The precipitate was again transferred into a narrow tube and dissolved in 0.5 ml N HCl, sealed and hydrolysed for 16 hours at 100 °C. Then the tube was opened and the hydrolysate was transferred to Whatman 2 chromatographic paper along with the following standards: D-glucose, D-galactose, L-fucose, D-glucosamine hydrochloride, L-desoxyglucose. The chromatogram was then developed in an ascendant way in a system of n-butanol-pyridine-benzene-water (5 : 3 : 1 : 2), whereby the developing was repeated three times. After drying the detection of the chromatogram was carried out, using $AgNO_3$ in acetone (7).

Experiment 5: 50 g of crushed bone were eluated with four times 50 ml 90% phenol at 30-minute intervals. We were unable to obtain a precipitate by addition of alcohol to phenol as reported by Morgan (23). Therefore the phenol extract was shaken with six times 25 ml distilled water. The extracts were combined and evaporated to a volume of 7.5 ml. Next the solution was subjected to precipitation with 96 % alcohol with Na_2SO_4, similarly as in the previous experiment. The precipitate was dissolved in distilled water and dialysed against distilled water for 16 hours. The dialysate was concentrated *IN VACUO* on a waterbath and transferred into a small tube and sufficient 1 N HCl was added to obtain a final concentration of 0.5 N HCl. The tube was sealed and the same procedure was used as in the previous experiment with the sugar hydrolysate.

RESULTS

Table 3 presents the results obtained in absorption tests with bone material from burial place IV. The technique described as method A was used, i. e. incubation of the bone material with antisera in a refrigerator or at room temperature. If we evaluate the results of tests according to common criteria, i. e. that only a drop with more than two titration grades is significant, we find that practically all the examined specimens would be classified as AB, except speciment No. 8 (0) and specimen No. 22 (B); when comparing the results obtained at the two different temperatures they do not differ much and in those instances where the specimens were tested by absorption tests at 4 °C and 18 °C (i. e. specimens 5, 10, 21, 22, 53, 100) the results are practically the same. Only in specimen No. 22, according to the criterion defined, the results differed and were B and AB respectively, depending on the incubation temperature.

Table 4 summarises the results obtained with bones from burial place I. The procedure used was different and corresponds to that described as method B. At first sight the results are more differentiated. Group AB was found 30 times, O 7 times, A six times and B three times. However, the finding of 30 AB groups in a total of 46 is very unlikely.

Table 5 presents the results of absorption tests carried out in a somewhat different way (method C); the results indicate that the elution fluid (physiological saline) after 48 hours incubation with bone material at 4 °C does not contain group active material. The remnant after absorption, i. e. bone material, preserved its inhibitory capacity in relation to antisera even after this period.

TABLE 3

Residual agglutinins present in anti-A and anti-B sera after their treatment with bone material (burial site IV)

Absorption temperature	Anti-serum	Control	1	2	3	4	5	6	7	8	10	12	13	14	15	17	18	19	21	22	33	39	40	41	41	42
4 °C	Anti-A	32		8		8	2			32	4	2		4	8		8		8	16	2			4	2	2
	Anti-B	32		8		8	4			32	4	4		4	8		2		4	2	8			2	2	4
18 °C	Anti-A	32	4		4		8	4	8		4		4			4		4	8	4		4	2			
	Anti-B	32	4		4		8	2	4		4		4			2		4	2	4		2	2			

Absorption temperature	Anti-serum	Control	44	45	48	50	51	53	63	64	68	69	74	77	79	79	80	81	85	88	90	92	95	97	99	100
4 °C	Anti-A	32	2		2	2	4	8	4		8		8		8	8		4	4	4	8		8	8		4
	Anti-B	32	4		8	8	4	4	4		4		2		4	4		4	0	4	8		8	4		4
18 °C	Anti-A	32		8				4		4		2		2			2					4			4	2
	Anti-B	32		4				8		4		2		2			4					4			8	0

TABLE 4

Residual agglutinins in anti-A and anti-B sera after their treatment with bone material (burial site I)

Antiserum	Control	1	6	10	13	20	21	26	28	30	34	35	36	37	42	44	46	51	52	54	56	65	66	68
Anti-A	32	2	8	4	0	4	4	16	8	8	8	32	8	8	8	4	2	8	8	4	4	8	16	8
Anti-B	32	4	4	4	2	4	8	32	16	8	8	16	16	8	8	8	16	8	4	8	4	8	8	16

Antiserum	Control	73	74	77	78	80	82	83	84	90	92	96	97	98	99	102	116	117	120	123	124	132	157	159
Anti-A	32	4	8	4	8	16	4	8	2	8	32	4	8	4	8	8	16	32	32	v4	8	16	8	16
Anti-B	32	8	16	8	4	16	4	8	4	8	16	8	16	8	8	8	8	16	16	8	8	16	8	8

TABLE 5

Results of absorption tests (eluate and eluted substrate tested)

Specimen	Eluate		Eluted substrate	
	Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B
Control	64	64	32	32
12/IV	64	64	2	8
15/IV	64	64	8	8
18/IV	64	32	8	8
20/IV	64	64	8	8
33/IV	32	64	8	8
42/IV	64	64	8	8
48/IV	64	64	16	16
63/IV	64	64	8	8
89/IV	64	64	8	8
90/IV	64	64	4	8
95/IV	64	64	4	8

TABLE 6

Repeated absorption with bone material in 3 independent tests

Specimen	1.		2.		3.	
	Antiserum		Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
Control	32	32	64	64	64	64
39/I	8	4	64	32	64	64
43/I	8	8	32	32	64	32
58/I	4	8	16	16	64	32
59/I	4	4	16	16	64	16
76/I	8	4	16	32	64	32
94/I	8	4	16	16	64	32
114/I	4	4	64	64	64	16
119/I	4	4	32	32	64	32
122/I	8	4	32	64	64	64

Next a series of reactions with repeated saturation of the same material was made.

Table 6 gives the results obtained by method B, whereby the same tests were made independently on three different days. The results indicate that the results did not agree.

TABLE 7

Repeated absorption tests with the same bone material-using in each test other antiserum portions

Specimen	1.		2.		3.	
	Antiserum		Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
Control	64	64	64	64	64	64
17/I	64	32	16	32	64	32
28/IV	32	32	16	32	64	32
29/IV	32	32	32	32	32	32
31/IV	16	32	32	32	32	16
46/IV	16	32	16	32	32	32
62/IV	16	32	32	16	32	16
66/IV	32	32	16	32	32	32
68/IV	16	32	32	32	32	16
74/IV	16	32	16	32	32	16
76/IV	32	32	16	32	32	32
83/IV	16	16	16	16	32	32
86/IV	32	32	32	64	32	16

Table 7 records the results of repeated absorption: the bone material was subjected to three absorption experiments, whereby always for the absorbed antiserum from every experiment a new antiserum (method D) was substituted. The results cannot be evaluated as in no instance a marked drop of titre was observed, as compared with controls.

Table 8 summarises another group of tests; there the same bone material was again repeatedly used for absorption of antisera which were titrated after each partial absorption (method E); in this case balanced antisera were not used and their potency was adjusted only by dilution with physiological saline to a titre 1 : 32; anti-A serum was diluted 1 : 35, anti-B serum 1 : 10. It cannot be ruled out that in this case the dilution factor played some part.

TABLE 8

Repeated absorption with homologous bone material in 4 independent tests

Specimen	1.		2.		3.		4.	
	Antiserum		Antiserum		Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
3/IV	16	32	4	16	4	32	8	32
4/IV	8	32	16	32	0	32	16	32
5/IV	16	32	8	32	0	32	16	32
14/IV	16	16	16	32	16	16	8	32
17/IV	16	32	16	16	8	32	16	16
44/IV	16	32	16	32	16	16	16	32
51/IV	32	16	16	32	8	16	16	32
79/IV	8	32	4	32	8	32	8	16
80/IV	8	32	16	32	4	32	4	32
81/IV	8	16	16	32	16	32	32	32
88/IV	4	32	4	16	2	32	8	32
97/IV	4	16	0	16	8	32	4	32

TABLE 9

Repeated absorption tests with homologous bone material

Specimen	1.		2.		3.	
	Antiserum		Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
Control	32	32	16	16	16	16
27/IV	4	8	4	4	0	0
34/IV	8	8	8	8	0	0
61/IV	4	4	4	4	0	0
75/IV	8	8	8	8	0	0
94/IV	8	16	8	8	0	0

Table 9 (method F) summarises results of absorption tests described as 1. and 2. in a table of two independent experiments and as 3., the results of titration of antisera obtained during the first and second experiment after absorption with new homologous bone material. While absorption carried out in the two independent experiments gave differentiated results, the mixture of antisera from these experiments after another absorption gave in all instances negative results. It thus cannot be ruled out that this result may be due to the destruction of antibodies in antisera resulting from the liberation of decomposition products from the bone.

Table 10 presents the results of analyses of soil specimens and some chemicals (minerals). The results of three independent experiments suggest that the substances used, particularly minerals, are not without a considerable influence on the results of absorption tests.

TABLE 10

Absorption of antisera anti-A and anti-B with soil samples and minerals in three independent tests

Sample	1.		2.		3.	
	Antiserum		Antiserum		Antiserum	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
Control	64	64	32	64	32	32
Find-stratum	16	16	16	32	16	16
1. niveaux	16	4	32	8	16	16
2. niveaux	32	32	16	32	32	16
Sample 40 cm under skeleton	64	32	32	16	16	16
Calcium-phosphate	0	0	8	8	8	8
Calcium-carbonate	4	4	0	2	32	8
Magnesium-phosphate	32	32	32	16	16	32

Table 11 presents the results of experiments with the absorption of anti-Rh (anti-D, anti-C and anti-E) sera, some of which were carried out repeatedly in independent tests. It is interesting that in some instances a considerable drop of antisera titres occurred, particularly in anti-D serum.

TABLE 11

Absorption tests with bone material using Rh antisera (3 independent tests)

Specimen	Anti-D			Anti-C			Anti-E		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
Control	128	128	256	256	256	128	256	128	64
2/IV		2			64			128	
8/IV	16	8	16	64	32	32	8	32	4
9/IV	8	8	8	32	32	32	8	32	8
13/IV	8	8		64	64		64	32	
19/IV	4	4	4	64	64	16	64	16	8
39/IV	4	16	32	128	64	32	64	64	64
40/IV	16	16		64	64		32	16	
41/IV	8	16		64	32		16	128	
74/IV	16	0	4	32	32	32	16	16	32
77/IV		8			32			64	
80/IV	8	4		256	64		64	64	
81/IV	16			32			32		
85/IV	8			64			32		
92/IV	4	64		128	256		64	128	
126/IV			16			16			32
131/I			16			32			128
134/I			16			32			32
137/I			16			64			16
143/I			32			32			32
162/I			16			32			16

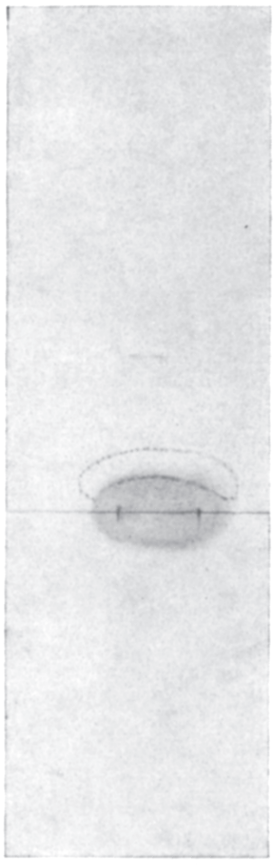

FIGURE 1

Electrophoreogram of eluate from bone material. Pyridine—acetic acid—water (10 : 4 : 450) buffer, pH 5.3, 9.3 V/cm, $2^1/_2$ hr.

Results of chemical analysis:

Experiment 1 revealed in bony tissue the presence of neutral amino acids *(see fig. 1).*

The first part of experiment 2 gave a negative reaction for reducing sugars. The second part of this experiment gave a positive reaction (reducing sugars were liberated by hydrolysis of the material). Free reducing sugars thus were not present in crushed bone.

Tests for the presence of protein (experiment 3) did not give a blue coloration but a bluish-green one. As the phenol reagent is not specific for proteins only (it reacts for instance also with uric acid, gua-

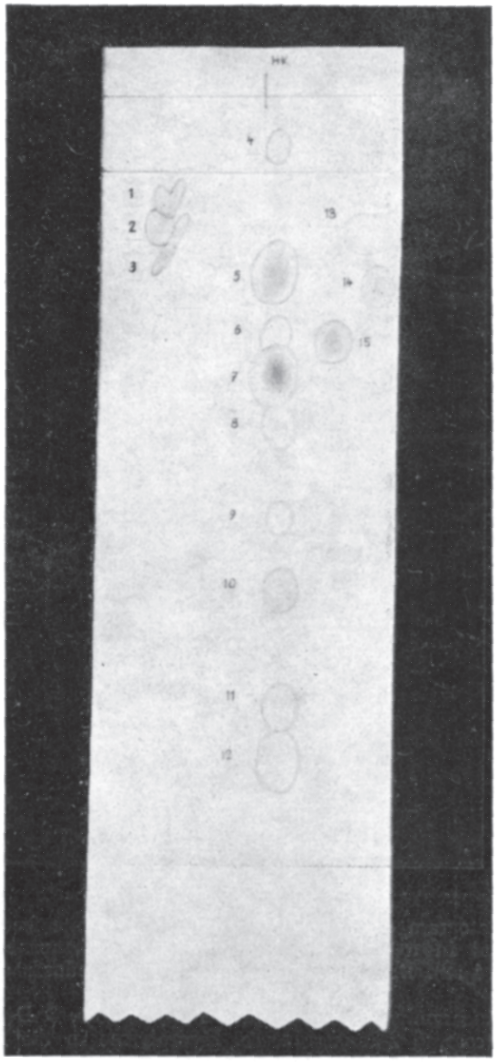

FIGURE 2

Electrophoreogram and chromatogram of water eluate from bone material. Electrophoresis was conducted in pyridine—acetic acid—water (10 : 4 : 450) buffer, pH 5.3, 9.3 V/cm, $2^1/_2$ hr. Chromatography performed in a system n-butanol—acetic acid—water (4 : 1 : 5).

1 lysine, 2 histidine, 3 arginine, 4 cystine, 5 serine and glycine, 6 threonine, 7 alanine, 8 proline, 9 tyrosine, 10 methionine, valine and tryptophan, 11 phenylalanine, 12 leucine, 13 cysteic acid, 14 aspartic acid, 15 glutamic acid. Ninhydrin detection.

nidine, hydrazine, etc.) from this experiment no conclusion on the presence of protein can be drawn. By chromatography (experiment 4a) in the crushed bone the presence of the folowing neutral amino acids was revealed: alanine, tyrosine, leucine, phenylalanine. Spots were also found which contained one or more of the following possible amino acids: valine-methionine-tryptophan and also serine-glycine. In the underlying soil (niveaux) by the same method the following amino acids were found: lysine, histidine, alanine, threonine, tyrosine, leucine, glutamic acid, a spot of valine-methionine-tryptophan and serine-glycine.

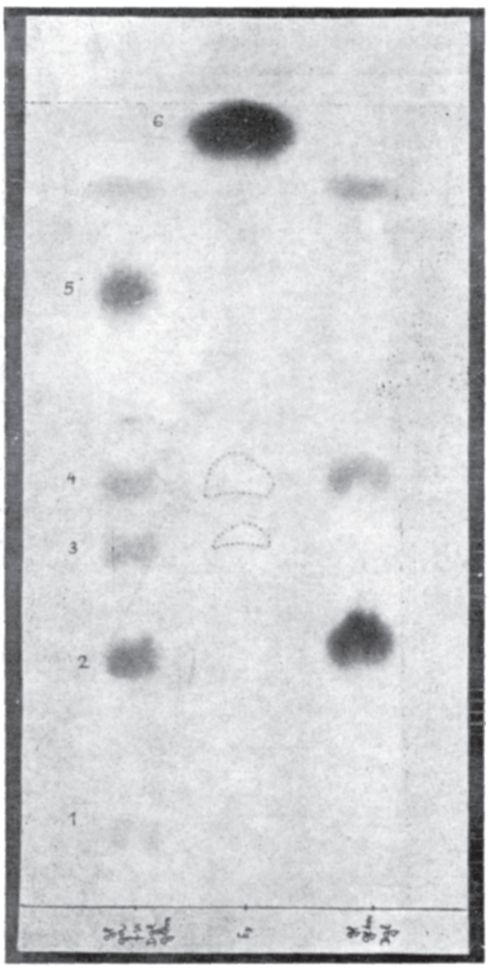

FIGURE 3

Chromatogram of hydrolysate of aqueous eluate from bone material in a system n-butanol—pyridine—benzene—water (5 : 3 : 1 : 2). Detection with $AgNO_3$. The standards are run on the sides.
1 D-glucosamine hydrochloride, 2 D-galactose, 3 D-glucose, 4 L-fucose, 5 2-desoxyglucose. Hydrolysate in the middle. 6 A non identified reducing sugar.

In the subsequent part of the experiment (4b alcohol precipitation) from the eluate of the crushed bone a protein-like substance was obtained. After its hydrolysis, made in order to analyse the amino acids, the presence of arginine, lysine, histidine, glutamic acid, aspartic acid, cysteic acid, cystine, serine and glycine, alanine, threonine, proline, tyrosine and a spot valine-methionine-tryptophan, phenylalanine, leucine *(fig. 2)* was obtained. The hydrolysis, made to detect sugar components, revealed that their concentration in the precipitate is small and therefore another experiment was carried with a larger amount of precipitate (experiment 5).

The amount of protein-like substance obtained from the soil by alcoholic precipitation was so small that it could be hydrolysed only for assessing the amino acids present. Practically the same amino acid spectrum was found as in crushed bone.

Experiment 5 revealed in the isolate the presence of a sugar *(fig. 3)* the R_F of which in the given system is greater than the R_F of desoxyglucose. For lack of standards it was not identified more closely. It seems that apart from this substance also glucose and perhaps also galactose are present in very small amounts.

DISCUSSION

The idea of providing evidence of blood group substances in paleoanthropological material, sometimes very old material, is very attractive. Therefore pioneer work in this field of research (B o y d, C a n d e l, M a t s o n) aroused much attention. Despite this the results of trials made so far are not quite convincing and in hitherto published work there are opinions calling for careful evaluation of the results of absorption tests (9, 14, 27, 28, 32).

Particularly reports which were published recently on the results of the assessment of groups, using the compact portion of bone, do not confirm that the tests were correct (14, 33); on the contrary, it appears that compact bone is not suitable for these tests. For instance Weinberg et al. (33) did not provide evidence, using absorption and elution tests, of the presence of group substances in cortical bone fragments, taken during surgical operations and stored for several weeks in a frozen state. On the other hand, in the spongiose portion of bone they were able to assess the blood group and the result was in agreement with the group assessed in the red blood cells. The spongiose portion of bone thus seems to be more promising, as during the life time of the individual it is in permanent contact with haematopoietic tissue and it can be thus assumed that remnants of the original material may have been preserved up to the present, particularly when the bone was protected during storage from external factors of the soil. In our work we devoted attention to tests for the presence of group specific substances AB and CDE in the spongy bone of preserved skeletons. Our results, however, do not permit a definite conclusion as to whether specific blood group substances were actually proved. In a number of differently modified tests used it was revealed that a certain inhibitory activity exists, particularly when suitable conditions are selected for the test; whether, however, the isoagglutinin inhibition is caused only by group specific substances of human origin or by contaminating substances of other origin, obviously cannot be decided. The use of the cancellous bone is also complicated by the same factors which make

the results of the tests with the compact portion of bone doubtful: for instance the possible presence of decomposition products of the organic bone matrix, the presence of antigenic components formed by soil factors, etc.

There is thus a number of circumstances which may influence the results of absorption tests. Also the selection and procedure of absorption tests are not without importance. This applies in particular to the most suitable method of preparation of crushed bone for the test. Mechanical crushing of the scraped out spongiosa, if possible to a very fine powder, is an essential prerequisite. The condition quoted in the literature (32), that the crushed material should not be exposed for too long to the action of the atmosphere, does not seem essential; despite this we tried to examine the material within a short time following its isolation. Some workers first wet the bone or rinse it and only then carry out the absorption tests. We used this method as well as direct combination of dry tissue with antiserum and the results did not differ much. It thus does not seem to be an important condition, though rinsing with physiological saline apparently does not leach the group active material from the crushed bone, as was proved by leaving the crushed bone in physiological saline (overnight in a refrigerator), the removed supernatant fluid being not active in the absorption test. This means that rinsing does not influence the liberation of group active substances which even after rinsing are still firmly bound to the bone substrate.

Different periods of incubation are given in the literature: in some tests we therefore incubated the substrate with antiserum for three hours, in others for a longer period (overnight) and the results did not differ markedly.

The amount of material used for saturation was in our tests either of the same volume or 0.7 g of wetted tissue were used for 0.6 ml antiserum. These conditions correspond roughly to the experience of other authors (9, 33).

To the selection of suitable antisera great attention must be paid. Non-immune sera are preferred and their titre is usually adjusted by dilution with physiological saline. In our experiments antisera with a balanced titre were used, selected in such a way that in the undiluted sera the corresponding anti-A and anti-B titres were equal. Then the same dilution could be used, i. e. the protein content of the sera was equal, and it can be assumed that also the content of antibody active globulins was balanced. This condition is important, as in the absorption an uneven decline of the titres could manifest itself, due to a disbalance between the reacting antibodies.

As far as the results of Rh types D, C and E are concerned, our findings are not very convincing: while in anti-D antisera we obtained practically in all tested specimens of bony tissue positive results, in anti-C and anti-E antisera only in several instances the presence of a group active substance could be assumed. Moreover, the reproducibility of results is not the same. So far nobody attempted to assess antigenic Rh substrate in this connection; only Gilbey et al. (18) tried to estimate in mummified muscular tissue D, C, E and c with a positive result.

While check-up examinations of soil specimens from the sites where the skeletons had been found did not reveal a marked drop of antibody titres when absorption tests were applied, it is certainly of interest that in this respect minerals like calcium phosphate and calcium carbonate are effective. From this it can be assumed that the presence of these minerals is not quite indifferent for the results of absorption tests. In conjunction with this Candel (9) mentioned that the non-specific adsorption of isoagglutinins by human bone can be also due to a high mineral content in the spongy bone. It is known that various substances (such as kaolin, coal, benzonite) can cause a reduction of isoagglutinin titres (32).

Absorption tests wits bony tissue from paleoanthropological material are, of course, handicapped by the fact that it is impossible to make parallel control tests. In this respect the experiments contributed little to confirm the convincing character of tests. For instance Thieme et al. (32) prepared synthetically old bone specimens: portions of the bones were kept for 2—3 years in stone jars in sandy soil; the results of the tests were not quite convincing, as from a total of 19 specimens only in ten the tests were correct. The physical and chemical conditions of this stored bones resemble, of course, to a very slight degree the conditions to which bone remnants are exposed for many centuries.

For detection of group substances in bone inhibition tests are used and the results are evaluated according to the decrease of the isoagglutinin titre. It is assumed that this drop is caused by a specific substrate. It was, however, revealed that this drop may be due also to non-specific reasons, on other grounds, than those hitherto mentioned. It may be brought about also by ubiquitous substances which closely resemble blood group substances and are of plant or animal origin and which may participate as contaminating substances in the isoagglutinin inhibition.

On the other hand, it must not be overlooked that certain bacterial enzymes may cause the decomposition of blood group antigens (26, 31). Transformations of blood group substrates under the influence of bacterial enzymes were also described, the transformation of one group into another (A and B resp. into O(H) Iseki et al. quoted 15). According to Springer et al. (30) however, direct enzymatic destruction of the specificities of A, B and O(H) substances by microorganisms is more frequent than the transformation of A, B into O(H). According to these authors the alkalinity and acidity of the soil is more important, as it exerts a more direct effect on the stability of group antigens; serologically specific glycoside bonds are very labile in acid, while hexosamines are more sensitive to the action even of weak alkalis.

Confidence in the possible proof of group substances is based also on the finding of organic material (incl. amino acids which are less stable than

group specific substances) in much older material. Abelson (1) for instance detected the presence of organic material in fossiles which were sufficiently isolated from environmental influences and reports also on the finding of amino acids (10 % of the contents) in bones immersed in asphalt for many centuries (La Brea tar pits in Los Angeles). In our case, however, amino acids found in aqueous eluates of crushed bone may come from the underlying soil though it is less probable that they should have penetrated into the centre of the bone. As regards the revealed mucoid this possibility can be refuted, as its amount in the soil was considerably smaller than the amount found in the crushed bone. We rather may assume the reverse process, i. e. diffusion of mucoid into the soil.

From what has been described, it is obvious that it is difficult to assess whether by inhibition tests with paleoanthropological bone material evidence is actually provided of the presence of active specific blood group substance. Further research will be needed to solve this problem adequately in future.

CONCLUSION

A number of serological and biochemical tests were carried out to elucidate in greater detail problems of whether in preserved material from skeletons from burial places in Mikulčice (Moravia — ČSSR) group specific substances AB or Rh (CDE) can be detected. The data assembled did not provide reliable results, as the reproducibility of tests was poor and control examinations revealed that the occurrence of non-specific results is too great to consider the results reliable. Biochemical findings revealed the presence of neutral amino acids and mucoproteins, which cannot, however, be considered without reserve as substances of human origin.

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