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Analysis of August rat liver for calcium, copper, iron, magnesium, manganese, molybdenum, potassium, sodium and zinc*

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The concentrations of Ca, Cu, Fe, Mg, Mn, Mo, K, Na and Zn in "August" pure line rat livers has been determined by emission spectroscopy, polarography and flame photometry. Differences were shown to exist between normal, regenerating and neoplastic livers, especially in their calcium, iron and manganese contents.

BERGEL, Everett, Martin & Webb (1957) described a semi-quantitative method of analysis of Wistar rat livers by emission spectrography. The present communication deals with the extension of these comparative analyses to include major elements and more refined methods and techniques, i.e., flame photometry with ion-exchange chromatography, polarography and emission spectrography; this time the livers of "August" rats (a genetically pure line strain) were used.

Animal material

The preparation and pre-treatment of tissue samples subjected to analysis were similar to those reported by Bergel & others (1957) except that after drying, the tissues were not submitted to a defatting process.

The following pooled normal livers from rats ("August" strain, fed on MILL HILL rat cake diet No. 44) represented our starting material for analytical procedures: foetal, new born, 5, 7, 10, 14, 17, 21, 24, 28, 35, 42 and 56 days old. In addition we had at our disposal, as pooled material: maternal livers from those mother animals which provided the foetal samples; regenerating livers, produced as described previously (hepatectomies by Miss E. Leuchars); the abnormal livers were produced by feeding 12 rats (6 weeks old) a diet of 10% protein with *p*-dimethylamino-azobenzene (600 mg/kg dry weight) for 4 months. Of 12 rat livers, 5 proved unsuitable, and the remaining 7 were submitted to histological examination. All the specimens consisted for the main part of tumour tissue, some of the tumours being of parenchymal cell origin and some of bile duct origin. Where surrounding liver tissue was also present, fatty degeneration or bile duct hyperplasia were evident. Details are as follows: Rat I. Multiple parenchymal-cell hepatomas and multiple cholangiomas. Rat II. Malignant cholangio-carcinoma. Rat III. Extensive bile duct hyperplasia and multiple cholangiomas. Rat IV. Malignant cholangiocarcinoma. Rat V. Multiple liver tumours, some of parenchymal cell origin and some of bile duct origin, also focal fatty infiltration. Rat VI. Large malignant parenchymal cell hepatoma. Rat VII. Areas of bile duct hyperplasia and two parenchymal cell hepatomas.

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* The second paper on major and minor metals in normal and abnormal tissues. The first paper was by Bergel, Everett & Webb (1957).

TABLE 1. ANALYTICAL RESULTS

Liver (age in days)	Dry wt (g)	Ash wt (g)	Ratio dry wt/ ash wt	Na (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Cu (ppm)	Mg (ppm)	Mn (ppm)	Mo (ppm)	Zn (ppm)
Foetal	0·4019	0·0251	16·0	5,630	13,750	1,250	290	19·65	415	3·37	0·272	463
5	7·4358	0·4431	16·9	3,580	11,540	886	201	30·2	349	2·53	0·475	430
7	8·7587	0·4439	19·8	3,920	9,080	832	263	83·2	450	1·31	0·69	414
10	9·3366	0·4808	19·4	4,050	8,750	824	247	64·3	480	1·09	0·34	202
14	10·2940	0·6638	15·5	3,100	12,280	1,225	165	100·0	670	1·99	0·61	284
17	13·6223	0·8976	15·2	3,230	7,880	1,052	303	99·7	885	1·73	0·92	257
21	13·7257	0·7140	17·1	2,980	6,720	672	213	97·8	360	1·17	0·76	216
24	12·9083	0·5187	24·8	2,950	5,640	625	131	26·3	254	1·15	0·50	210
28	11·3644	0·5190	21·8	2,910	10,120	714	253	27·1	335	1·91	1·23	202
35	12·9491	0·5427	24·0	2,590	8,120	624	195	18·6	338	1·17	1·18	163
42	15·9080	0·8680	18·3	2,330	10,130	900	202	38·0	509	0·88	1·16	328
49	7·8700	0·3301	23·8	2,590	6,300	566	160	55·0	404	1·89	0·86	113
56	17·8309	0·6998	25·5	2,270	8,230	725	187	22·1	432	1·57	1·22	137
Maternal* ..	10·4855	0·7718	15·4	1,420	9,070	741	249	24·7	442	1·24	1·62	175
Regenerating†	7·3778	0·3806	19·4	6,620	5,930	592	173	21·9	788	1·15	1·42	248
Tumour‡ (6 month)												
Rat No. I ..	10·9227	0·6571	16·6	3,650	9,920	2,010	361	20·8	320	4·34	0·94	127
Rat No. II ..	11·6438	0·6047	19·3	1,960	6,730	713	456	33·2	394	1·24	1·06	155
Rat No. III ..	7·4280	0·3942	18·85	3,050	5,830	1,340	392	19·65	403	4·68	1·83	101
Rat No. IV ..	10·2544	0·5968	17·2	3,920	5,800	1,745	407	54·4	459	3·14	1·34	134
Rat No. V ..	13·4394	0·6755	19·8	3,570	8,320	1,350	404	54·5	223	4·66	0·91	138
Rat No. VI ..	8·2642	0·4563	18·2	3,240	9,320	1,375	357	57·0	710	4·40	1·42	149
Rat No. VII ..	12·6496	0·7482	16·9	3,080	5,910	2,170	438	39·7	398	2·84	0·73	160

* Maternal livers from pregnant rats.
† Regenerating liver. Removal of one lobe and then allowed to regenerate (5 days).
‡ Tumours arising from rats fed on a diet containing a carcinogenic Azo dye.

All results are quoted in ppm of dry tissue.

Analytical methods

Sodium, potassium and calcium were determined using the Evans Electroselenium Flamephotometer; iron, copper, zinc, manganese and molybdenum using the Southern Instruments K1000 Polarograph and magnesium using the Hilger Medium Quartz Spectrograph.

FLAME PHOTOMETRY

(*a*) Sodium and potassium. The ash (*ca.* 10 mg) was dissolved in 0·5 ml of concentrated hydrochloric acid (Analar) and the solution made up to 10 ml (1 mg/ml) with water. This solution was diluted × 100 for potassium and × 1,000 for the sodium and then aspirated into the flame of the photometer. Standard working curves were established, using a series of prepared standard solutions containing known quantities of both elements. It was found previously that by using the dilutions mentioned, interference from other elements in the flame was negligible.

(*b*) Calcium. The method described by Hemingway (1956) was used. 1 ml of the solution containing 1 mg/ml (solution as used previously for Na and K) was passed through a column containing a cation-exchange resin (Amberlite IR-120 (H) ground to the recommended size), to retain the calcium, free of phosphate. The column was washed with water and the calcium eluted with 5N nitric acid (Analar), until 10 ml of eluate was obtained which was then aspirated into the flame. Standard working curves were obtained by passing through the columns solutions, containing known amounts of calcium; they were eluted with 5N nitric acid.

POLAROGRAPHY

(*a*) Iron. The method as described by Hetman (1959) was applied. A known amount of ash was dissolved in concentrated hydrochloric acid, and the solution diluted with water. 5 ml of this solution was introduced into a 10 ml graduated flask, then the base electrolyte, consisting of 2 ml 10M sodium hydroxide, 1 ml M sucrose and 1 ml of a saturated solution of EDTA was added and the total brought up to the mark with water. The polarogram was recorded on a 5 ml aliquot, after de-aerating with N_2 for 3 min, applying cathodic reduction and using a Hg pool as reference electrode. Standards were determined, using the above procedure, and working curves established.

(*b*) Copper. For this metal the method of Carruthers & Suntzeff (1945) was used: a known amount of ash was dissolved in 1 ml of 0·1N hydrochloric acid and 1–2 drops of concentrated nitric acid was added to oxidise the iron to the ferric state. The solution was evaporated to dryness on a water-bath, the residue dissolved in 2 ml of 0·1N potassium thiocyanate and the solution polarographed after removal of the oxygen.

(*c*) Manganese. Following the method, described by Hamamoto (1934), a known amount of ash was dissolved in concentrated hydrochloric acid. The solution was gently heated, allowed to cool and sodium carbonate was added to give a weakly acidic reaction. The addition of saturated sodium acetate and heating gave a basic iron acetate as precipitate which was filtered off and discarded. To the filtrate was added a

small crystal of potassium chlorate which was allowed to dissolve; then 20 ml of 3N sodium hydroxide were added. On heating, the manganese was separated as $Mn(OH)_2$ and MnO(OH), the precipitate was filtered and ashed in a crucible. This ash was converted to the chloride, dissolved in 2 ml of 0·001N lithium chloride and polarographed after the removal of the oxygen. Standard working curves were established, using the same procedure.

(*d*) Molybdenum. Following the method described by Jones (1954) a known amount of ash was dissolved in concentrated hydrochloric acid and 0·1 ml sulphuric acid, and the solution diluted with water to 10 ml. 2 ml of a 2% ethanolic benzoin oxime solution was added and the total extracted with chloroform; the extracts were combined and the chloroform removed by evaporation. The residue was decomposed with acids, the solution cooled, and to it 4·8 ml of 1M sodium perchlorate were added. The final volume was polarographed after removal of oxygen.

(*e*) Zinc. The method of Cholak, Hubbard & Burkey (1943) was applied. A known amount of ash was dissolved in 0·5 ml concentrated hydrochloric acid and to the solution 30 ml of 20% ammonium citrate added with 4 drops of 0·1% thymol blue in water. The total was adjusted to pH 9·5 with ammonia; 4 ml of 1·25% aqueous solution of sodium diethyldithiocarbamate was added and a final volume of 100 ml reached by dilution with water. The solution was shaken with 5 ml portions of a chloroform solution of di-β-naphthylthiocarbazone (200 mg in 990 ml of chloroform and 10 ml of ethanol), until the original blue-green colour remained unchanged. The chloroform extract was washed with 50 ml of water and extracted by shaking it with 50 ml of 0·2N hydrochloric acid. The aqueous solution was evaporated to a volume of 1–2 ml, cooled and its pH adjusted to the change in methyl red with ammonia. The volume was brought to 5 ml with water, de-aerated with N_2 and polarographed. Standard solutions were made, using this procedure, and calibration curves established.

EMISSION SPECTROGRAPHY

Magnesium. A known amount of ash was dissolved in hydrochloric acid (conc.) and the solution diluted with water. An aliquot (0·1 ml) was evaporated on flat top 6·5 mm diameter graphite electrodes (National Carbon Co., U.S.A.) which had previously been dipped into a light petroleum solution of 1% apiezon grease to render them non-porous. The electrodes were arced for 45 sec at 9 A and the spectrograms recorded on Kodak Photoscript B10 plates. The density of the spectral line, Mg 2783, was measured using a Hilger microphotometer and correction was made for "background" in the usual manner. Seidl density values (Black, 1952) were used and the concentration read from a working curve. Standard working curves had been established, using known amounts of magnesium added to a synthetic base prepared by Messrs. Johnson Matthey, London, described by Bergel & others (1957).

Water for all determinations had been passed through an Elgastat Deioniser (type B102) before use.

Results and discussion

Sodium. Inside the normal liver group the sodium content decreased from 5,630 ppm in foetal livers to an average of 2,400 ppm in adult livers. The maternal livers showed a further reduction to 1,400 ppm while the first sample of regeneration livers apparently had a high level of 6,620 ppm. Amongst the seven hepatomas the content averaged 3,400 ppm.

As the high sodium content of the first sample of regenerating livers was rather surprising as compared with the average content (over the whole age range), a further six control livers with corresponding regenerating livers were analysed. When matched pairs from individual rats, i.e., normal and regenerating livers from the same animals were analysed, the regenerating group averaged 3,150 ppm, whereas the control group averaged 3,015 ppm. It was therefore concluded that comparisons of values must be between the same age groups.

Calcium. Normal livers averaged 650 ppm, whereas calcium in the hepatomas increased in content to 1,600 ppm. This is in direct contrast to the values found by Kishi, Fuitwara & Nakahara (1937) who by using a gravimetric method found that in transplantable rat hepatomas the calcium values decreased. Delong, Coman & Ziedman (1950) also found that the calcium values were low in human intestinal cancers.

In both findings the reference tissue did not bear so close a relationship to the tumour tissue as did our tissues. For instance, in the case of the human intestinal cancers the adjacent normal mucosa was used as the reference tissue; whilst in the other case the tissue was a "transplantable" hepatoma (which after serial transplantation no longer bears much relationship to the original source from which it derived). The present work on the determination of calcium will be extended to include a variety of primary and transplantable experimental tumours including cellular fractions together with pertinent reference tissue.

Potassium. Normal liver decreased in content from 13,700 ppm in foetal livers to an adult level of 7,000 ppm. There was no significant difference in the content of the hepatomas in comparison with those of the adult rat liver, but with regards the foetal livers the values were lower.

Copper. During the suckling period in the life of the rats the copper content of the livers was increased, as reported (Lorenzen & Smith, 1947), and declined to a normal level to 25 ppm after weaning. In five out of the seven hepatomas studied the values were above the normal average, and in three of these the levels were of the order of 55 ppm. These figures are similar to those given by Bergel & others (1957) which were obtained by emission spectrography whereas the present figures were obtained by polarography.

Iron. In the "normal" liver groups the iron values were 200 ppm over the whole age range. This figure is very similar to that given by Bergel & others (1957). The content of the tumorous tissue was 400 ppm. This rise in value was also reported in that communication where attention was drawn to the possible fluctuation of iron due to the presence or

absence of residual blood. It should be pointed out, however, that there appears to be a genuine rise in the Fe content of hepatomas.

Magnesium. The 788 ppm of magnesium found in the regenerating liver appears to be high but is within the values of "normal" livers, the highest figure being 885 ppm. No significant differences were found, when the "normal" values were compared with those of the hepatomas.

Zinc. Zn levels decreased from 460 ppm to 210 ppm from foetal to adult livers. On the whole these figures agree with the overall pattern given by Bergel & others (1957), although the plateau is on a higher level. This could be accounted for by the change of the analytical method to a polarographic one. There were no significant differences between the Zn values of the neoplastic tissues and those of the adult "normal" livers.

Manganese. The increased manganese content found in the hepatomas as compared with the content of normal livers is one of the outstanding features of this study. In the previous communication where livers from the Wistar hybrid rat were used the values for the liver tumours were similar to those of the later age groups.

Molybdenum. The previously found continual increase in Mo content from foetal to adult rat livers (Bergel & others, 1957) was confirmed in this present study, 0·272 ppm to 1·24 ppm. The Mo content of the regenerating liver of 1·42 ppm was not significantly different from that of the adult livers. Previously no Mo could be detected by the use of emission spectra in the regenerating livers.

Histograms for all these elements were prepared and are kept for inspection at our address.

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