RESEARCH PAPERS

CELLULAR CONSTITUENTS. MAJOR AND MINOR METALS IN NORMAL AND ABNORMAL TISSUES

PART I. ANALYSIS OF WISTAR RAT LIVERS FOR COPPER, IRON, MAGNESIUM, MANGANESE, MOLYBDENUM AND ZINC

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Assessment of the usefulness of emission spectrography for comparative analyses of six elements, Cu, Fe, Mg, Mn, Mo and Zn, in rat liver has been made. It has uncovered the presence or absence of differences in the concentrations of these metals in livers between age groups, animals on different diets, pregnant animals and male animals with normal, regenerating and tumourous organs. A discernible trend in concentrations of certain elements from the embryonic tissue to that of the adult animal emerged. In some instances the abnormal and regenerating materials appeared to carry lower or higher amounts of the element compared with normal livers. With molybdenum, the livers of embryonic, newborn and young animals had low concentrations, slowly increasing; the results obtained with older animals formed a higher level rugged plateau.

COMPARATIVE analyses of normal and abnormal tissues or cells for organic constituents and for levels of enzyme activities have been carried out over a number of years in many laboratories[1]. Estimation of nucleic acids, proteins, amino acids, lipids, carbohydrates, vitamins, coenzymes, hormones, etc. have been undertaken in embryonic, regenerating, adult and corresponding tumourous material. While so far usually only quantitative and not qualitative differences between the contents of various comparable tissues have been found, these observations are helping towards a greater understanding of the biochemistry of growth, development and cancer.

More recent work has attempted to find more experimental support for the hypothesis[2] that induction of malignancy is due, at least in part to loss of, or change in essential proteins. Apart from the studies of the Millers[3] with azobenzenes, Weiler[4] from an immuno-chemical viewpoint established that the feeding to animals of butter yellow (4-dimethylamino-azobenzene) which leads to the development of hepatomas, causes also a loss of a liver antigen. He demonstrated later[5] a similar antigen loss in the kidney of the male hamster after insertion of stilboestrol pellets in the flank, producing over a period tumours[6] in that organ. Bhargava and Heidelberger[7] showed that 1:2:5:6-dibenzanthracene, a carcinogenic polycyclic hydrocarbon, in form of an oxidation product combined to a certain extent with the skin-proteins of mice which had been treated with the agent.

The chemical nature of the changed or partly deleted proteins has not been established up to now. But estimations of enzyme activities have shown that some of these levels are lower in tumours than in normal tissues, although they fall rarely to zero. To quote one example, the livers of animals treated with butter yellow according to Westerfeld, Richert and Hilfinger[8] have a diminished xanthine oxidase activity compared with those of untreated animals. This enzyme, which contains flavin adenine nucleotide, molybdenum and iron, is being submitted to a chemical study by Bergel, Bray and colleagues[9,10] and has been the subject of analytical investigations by Lewin[11]. He estimated the level of xanthine oxidase activity in the breast tissue of mice belonging to low (C−) and high (C+) tumour strains, and found that it fell per cell from that in the C− mice, via that in non-tumourous tissue of the C+ mice, to a low value in the breast carcinoma itself. Another aspect of changes of proteins and related compounds, this time in the cell membrane, was uncovered by Ambrose, James and Lowick[12] who found that the surface charge of tumour cells was of a more negative character compared with normal cells. Before then DeLong, Coman and Zeidman[13] had drawn attention to the loss of mutual adhesiveness between malignant cells and put forward the suggestion that this was due to a deficiency in calcium in the cell membrane.

These few examples give additional impetus to the idea that tissue or cell analysis in the field of cancer research ought to include estimation of major and minor mineral constituents. Many investigations have been made to correlate the content of trace elements in water, soil, plants and animals[14–16] and, to establish their general role in nutrition (cf. Underwood[17]). Lately, Tipton and colleagues[18,19], Koch, Smith, Shimp and Connor[20] and Stitch, and Sowden and Stitch[21,22] have commenced studies on the concentration of certain metals in human organs. Their exploratory researches leading to semiquantitative data should be contrasted with work aiming at the purification and characterisation of metal-carrying cellular constituents. One aspect of recent investigations on metalloflavoproteins has been mentioned before[9,10,23]. Systematic studies by Vallee and colleagues[24] resulted in the discovery that zinc formed an integral part of a number of dehydrogenases.

In other instances, such as the pyridoxal-pyridoxamine enzymes, and many more, the role of metals as activators through chelation of coenzyme, substrate and, maybe, apoenzyme has been established. Not yet fully confirmed is the possibility, as proposed by Kirby[26,27] and followed up by us in collaboration with him, that metals may participate in the bonding of deoxyribonucleic acid to proteins.

For our work, as presented in this paper, we have chosen an experimental approach which amounts to a restriction of the analytical procedures, in this instance emission spectrography, to one organ of a defined animal species of different ages and of known nutritional status, and to a small number of elements, namely Cu, Fe, Mg, Mn, Mo and Zn. In this way we were hoping to compensate for the numerous technical difficulties in obtaining significant results. Apart from the problem of selecting

normal and abnormal tissues for reliable comparison, a problem which we temporarily solved by concentrating on livers, there is the complication through variations of tissues in contents of fat, connective material, residual blood, necrotic or inflamed parts. Moreover, there remains the decision of chosing a practical base line, may it be wet or dry weight of the organ, its total nitrogen or phosphorus content or its cellularity, on which one can calculate the analytical data. Thus we were less expecting "absolute" values but more hoping for disclosure of certain trends in the changes of metal levels from one age group to the next, and in animals fed with carcinogenic agents or having a regenerating organ. Consequently, leads might emerge which could be followed by assessment of coenzyme or enzyme contents, or of metal-containing cellular components of a still undetermined functional character.

Animal Material

The livers of Wistar rats, bred at the Chester Beatty Research Institute and killed by breaking the neck, were removed immediately after death and pooled, so that sufficient material for analysis was available and the effect of variations between individual livers was diminished. Throughout, metal tools were avoided and glass knives and rods substituted. The following groups of livers (not perfused, except one tumourous liver) were used.

(1) Normal livers from male rats at different age levels and fed on a 14 per cent special cake nuts diet (North-Eastern Agricultural Co-operative Society, Ltd., Aberdeen). (*a*) 127 foetal livers for four samples; (*b*) 50 new-born for two samples; (*c*) 30 one-week old; (*d*) 30 two-weeks old; (*e*) 25 three-weeks old; (*f*) 21 four-weeks old; (*g*) 24 five-weeks old; (*h*) 30 six-weeks old; (*i*) 24 seven-weeks old; (*j*) 12 eight-weeks old; (*k*) 12 sixteen-weeks old; (*c*) to (*k*) one sample each.

(2) Normal livers (20 for three samples) from pregnant rats, 16 to 20 weeks old, fed on rat cakes and kitchen scraps; these animals were used also as a source of the embryos whose foetal livers were mentioned under (1*a*).

(3) Normal livers from rats fed on rat cake and kitchen scraps (*a*) 198 male for seven samples, 4 to 8 weeks old; (*b*) 100 for four samples 8 weeks old.

(4) Regenerating livers (8 for two samples) from rats, 8 weeks old, previously operated on for partial hepatectomy (removal of one lobe of the liver), the organ then being allowed to regenerate for 5 and 8 days respectively. The operations were carried out by Dr. Sheila Doak, to whom we are greatly indebted.

(5) Tumour tissue, excised from livers (7 for 7 samples, one perfused) of rats fed for thirteen to fourteen months on a diet with 20 per cent protein and 0·42 per cent of methyl butter yellow (4-dimethylamino-3′-methyl-azobenzene). The types of tumour present could not be accurately distinguished; they consisted mostly of hepatomas but also some cholangiomas and adenocarcinomas. As Price, Harman, Miller and Miller[28] have expressed the opinion that usually there is a gradual transition

from one type of abnormal tissue to the other, we refrained from classifying histologically the malignant liver-parts, and shall refer to them as "liver tumours".

Analytical Methods

(*a*) *Ashing of biological material.* Each group of pooled livers was weighed ("wet weight") and then dried in an electric oven at 110° for 24 hours. This material was powdered by grinding it in a glass mortar with glass pestle and dried at 110° to constant weight ("dry weight"). The powder was subsequently defatted by extraction in a soxhlet with absolute ether. The extracted residue was freed from solvent *in vacuo* with warming up to 50°. The product, weighed for "dry-defatted weight" ("W_{DD}") was incinerated in a "Vitreosil" crucible, placed in an electrically-heated muffle furnace at temperatures reaching 435° ± 1° inside 3 hours[15], when the heating was continued for another 7 hours. After cooling, the crucible was removed and its contents ground with an agate pestle. A few crystals of spectroscopically pure ammonium nitrate were mixed with the powder and the heating in the furnace continued at 435 ± 1° for 24 hours. The resulting colourless ash was weighed ("ash weight", "W_A") and stored in polythene specimen tubes until submitted to emission spectrographic analysis.

(*b*) *Emission spectrography.* The ash (*ca.* 20 mg.) was mixed with an equal weight of pure carbon powder and the mixture compressed into the bore of a Jelfke electrode made from spectrographically pure carbon rod to the following standard specification: external diameter of electrode, 3·0 mm.; internal diameter and depth of longitudinal bore, 0·8 mm. and 8·0 mm., respectively.

Four replicate spectrograms of each sample were obtained by the cathode layer arc technique (cf. Mitchell[14]) using a Hilger Large Quartz Spectrograph (E.492) and a 9 amp. D.C. arc. The external optical train consisted of a condensing lens and step filter (ratio 1 : 1·7). The lens focussed the cathode layer on the collimator of the spectrograph, and the spectrograms were recorded on Kodak Photoscript B.10 plates over the range 2700 to 4800 Å. The samples were burned to completion in the arc; this required a 200 second exposure.

The wavelengths of the lines used for the estimation of each element are included in Table I. The density of the spectral lines on the sample plates were measured using a Hilger microphotometer, correction being made for "background" in the normal manner. The concentration of each element sought was estimated in a manner similar to that described by Mitchell[14], with the exception that Seidl density values[29] were used instead of normal density values, the concentration being read graphically from a working curve correlating log concentration in parts per million of ash (log W_{E1}) and log per cent relative density of the spectral line.

The mean working curves were established from the line densities on replicate spectrograms given by a series of synthetically prepared standards containing known quantities of the elements sought. Each standard was spectrographed four times.

TABLE I

WAVELENGTHS OF LINES USED AND REPRODUCIBILITY OF RESULTS IN EMISSION SPECTROGRAPHY

Element and wavelength (A)	A		B	
	Known conc. in standard (p.p.m.)	Rel. standard deviation ± per cent	Mean conc. in sample (p.p.m.)	Rel. standard deviation ± per cent
Cu 3274	100	9·5	235	8·4
	316	8·4	386	6·6
	1000	2·8	509	7·8
	3162	6·5	1390	8·3
Fe 3059	1000	7·35	2560	21·1
	3162	7·98	6270	9·73
	10,000	6·54	7810	8·96
	31,620	7·05	20,200	10·89
	100,000	13·06	45,200	8·63
Mg 2783	1000	10·6	1040	15·1
	31,620	17·4	13,100	11·8
			16,000	9·9
Mn 2801	31·6	5·4	38	2·9
	100	4·5	55	5·8
	316	6·0	80	6·6
Mo 3170	3·2	15·8	7	13·8
	10·0	22·2	24	15·6
	31·6	11·4	34	18·8
	100	10·1	45	9·0
Zn 3345	3162	17·8	2450	12·1
	10,000	10·6	3250	10·9
			4830	12·7

The synthetic base, to which the elements were added in appropriate logarithmic proportions, was prepared by Messrs. Johnson and Matthey, London, by fusing the following mixture: KH_2PO_4 (21·0 g.); NaH_2PO_4 (4·96 g.); $CaCO_3$ (0·37 g.); Na_2CO_3 (0·42 g.); $NaNO_3$ (0·16 g.); NaCl (1·64 g.) and $(NH_4)_2SO_4$ (0·28 g.). The composition of this mixture was based on results of a bulk analysis of ashed rat organs, kindly undertaken by J. F. Harringshaw and L. S. Theobald of Imperial College, London, who found:—K_2O, 29·93; Na_2O, 8·19; CaO, 0·82; MgO, 2·15; Fe_2O_3, 0·04; SiO_2, 0·11; P_2O_5, 48·21; SO_3, 0·69; CO_2, 0·44; Al_2O_3, 0·02; N_2O_5, 0·4; NaCl, 2·97; H_2O, 1·02 per cent. Organic matter (containing N and/or O, 5·13 per cent); total, 100·12 per cent.

(*c*) *The reproducibility of the estimations.* As previously mentioned, the present examination has been concerned only with the estimation of copper, iron, magnesium, manganese, molybdenum and zinc. Figures for the reproducibility of the results for those elements over the range of concentrations encountered are given in Table I.

Reproducibilities were estimated using both synthetically prepared standards (A) and the results obtained on selected samples (B). For the former, the reproducibility is given as the relative standard deviation obtained by expressing the standard deviation of four estimations as per cent of the known content of the standard. With the samples (B), the standard deviations are expressed as per cent of the mean of four replicate estimations.

The significance of these figures is that statistically the limits of the relative standard deviation should be exceeded only 1 in 3 times by

chance. Data A and B show relatively good agreement, and in view of the fact that the samples represented in B are those which showed maximum variation, it is considered that a satisfactory degree of reproducibility has been achieved with the spectrographic method employed.

Results and Discussion

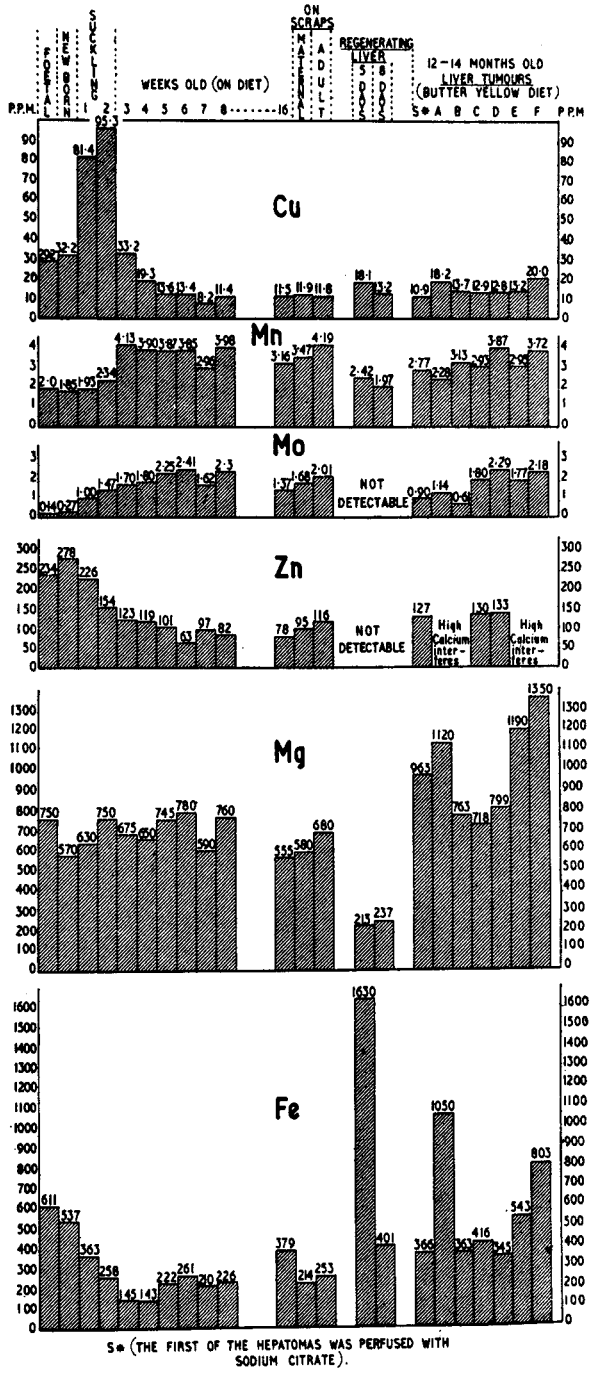
In a preliminary spectrographic survey of 30 elements the following were not detected under our experimental conditions: Be, Bi, Cd, Co, Cs, Ga, Ge, Hg, In, Li, Sb and Tl. While Rb was a contaminant of the synthetic base (cf. p. 526), Ag, Ni, Sn and Zr were found to be present in small amounts in certain samples only, and Ba, Cr and Pb seemed to form genuine constituents of some of the livers. Our more extensive studies were restricted to six elements, namely, Cu, Fe, Mg, Mn, Mo and Zn, which are known to play an important role as part of enzymes or as their activators.

The results are reported in Table II and Histogram I, where by using different scales for the various metals and averaged values for some groups, the changes are visually summarised. The data are expressed as μg. of element per g. of dried defatted tissue (W_{DD}) or p.p.m., calculated from the ratio W_A/W_{DD} (as given in Table II, column 1) and multiplied by W_{E1} where $W_{E1} = \mu$g. of element per g. of ash as obtained from the photometric values by the method mentioned above.

Copper (*cf.* Underwood[17], pp. 63–74). In normal livers the content increases suddenly from that of the embryos and newborn animals (*cf.* Lorenzen and Smith[30]) to about treble their amount in the one and two weeks old rats which are suckling at the time. The reason for this rise is rather obscure, particularly if one considers the low copper content of milk reported in the literature[31]. Our observation confirms that of Brückmann and Zondek[32], who have found a peak in copper concentrations in rat livers between 10 to 15 days after birth. Like these authors we established that in our animals, fed on a controlled diet, the copper content declined during the period from the 3rd to 16th week. Animals, 4 to 8 weeks old and pregnant rats 16 to 20 weeks old, both groups fed on kitchen scraps, did not differ very much from the former (*cf.* Ashikawa, Smith and Helwig[33]). The "liver tumours" from rats over 12 months old and the regenerating livers, after 5 and 8 days regeneration show only a slight increase compared with the organs of all adult animals mentioned above.

Iron (*cf.* Underwood[17], pp. 26–33). The level in tissues of this major element is, for obvious reasons, subject to fluctuations by the presence or absence of residual blood. Attention is therefore drawn only to the higher values noted in foetal livers of embryos and newborn animals, in liver tumours and in the regenerating organ. The average iron content of all other livers (21 groups) given in Table II is 236 p.p.m.

Magnesium. Figures for this other major element, so far obtained, are of restricted significance without the corresponding data for calcium. However, the values for the regenerating organ seem to be genuinely lower and the average for the liver tumours (986 p.p.m. from 7 samples) appear to be higher than the average of all other livers, namely 627 p.p.m.



HISTOGRAM 1. Individual analytical results of samples.

TABLE II

Individual analytical results of all samples

Age	Ratio	Copper		Iron		Magnesium		Manganese		Molybdenum		Zinc	
	W_A/W_{DD}	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample	p.p.m. in ash	p.p.m. in sample
Foetal	0·056	509	28·8	16,400	928	13,700	776	32	1·81	3	0·17	4260	241
	0·077	524	33·1	8540	658	13,400	847	32	2·02	2	0·13	2760	174
	0·063	470	29·7	6600	417	11,300	715	33	2·08	2	0·13	3370	213
	0·072	351	25·2	6150	441	9380	673	30	2·15	2	0·14	4300	308
New born	0·047	676	31·9	12,300	581	12,600	595	38	1·79	7	0·33	6510	308
	0·055	599	32·9	10,150	557	9830	540	35	1·92	4	0·22	5510	302
1 week	0·059	1390	81·3	6210	363	10,800	632	33	1·93	17	1·00	4350	255
2 weeks	0·055	1750	95·3	4730	258	13,800	752	43	2·34	27	1·47	4150	228
3 weeks*	0·057	588	33·2	2560	145	12,000	679	73	4·13	30	1·70	2820	159
4 weeks*	0·050	386	19·3	2850	143	13,000	650	78	3·90	36	1·80	2470	123
5 weeks*	0·049	278	13·6	4520	222	15,200	745	79	3·87	46	2·25	2430	119
6 weeks*	0·051	262	13·4	5080	261	15,200	780	75	3·85	47	2·41	1980	101
7 weeks*	0·041	205	8·3	5170	210	14,600	591	73	2·96	40	1·62	1560	63
8 weeks*	0·051	224	11·4	4430	226	14,900	760	78	3·98	45	2·30	1890	97
16 weeks*	0·045	252	11·5	8390	379	12,100	553	69	3·16	30	1·37	1790	82
4 weeks†	0·052	236	12·2	2450	126	10,700	551	67	3·45	34	1·75	1930	100
5 weeks†	0·043	241	10·2	3340	142	11,100	472	67	2·84	34	1·44	2240	95
6 weeks†	0·038	228	8·68	4580	175	13,000	495	77	2·93	34	1·29	2100	80
7 weeks†	0·046	215	9·95	5190	240	13,100	605	79	3·65	35	1·62	2170	100
8 weeks†	0·043	204	8·8	9800	422	12,700	547	67	2·88	28	1·23	1510	65
8 weeks‡	0·049	207	10·0	5530	268	14,400	699	82	3·98	42	2·04	2130	103
8 weeks‡	0·053	193	10·2	3750	199	12,000	635	82	4·35	32	1·70	2000	106
8 weeks‡	0·051	237	12·0	4700	238	11,800	596	86	4·35	39	1·97	2260	114
8 weeks‡	0·048	268	13·0	6340	307	14,600	705	84	4·06	43	2·08	2970	143
16–20 weeks§ ..	0·046	212	9·7	4050	186	11,300	520	68	3·13	34	1·56	2110	97
	0·046	235	10·8	4230	195	12,400	571	75	3·45	34	1·56	2060	95
	0·051	301	15·2	5110	259	12,600	639	76	3·85	38	1·92	1820	92
5 days¶	0·055	329	18·1	29,600	1630	3870	213	44	2·42	Nd	Nd	Nd	Nd
8 days¶	0·052	229	13·2	6940	401	4100	237	34	1·97	Nd	Nd	Nd	Nd
12–14 months** ..	0·096††	157	10·9	3800	366	13,900	963	40	2·77	13	0·90	1840	127
	0·076	240	18·2	13,800	1050	14,700	1120	30	2·28	15	1·14	Ca	High
	0·055	250	13·7	6620	363	13,900	763	57	3·13	11	0·61		
	0·047	273	12·9	8810	416	15,200	718	62	2·93	38	1·80	2750	130
	0·051	251	12·8	6770	345	15,700	799	76	3·87	45	2·29	2620	133
	0·074	179	13·2	7370	543	16,200	1190	40	2·95	24	1·77	Ca	High
	0·091	220	20·0	8880	803	14,900	1350	41	3·72	24	2·18	Ca	High

* Rats were fed on a controlled diet.
† Rats were fed on a normal diet plus kitchen scraps.
‡ Rats were fed on a normal diet plus kitchen scraps and the livers were pooled (four).
§ Maternal livers from pregnant rats.
¶ Regenerating livers.
** Tumours arising from rats fed on a diet containing a carcinogenic azo-dye.
†† Perfused with sodium citrate.

Manganese (see Underwood[17], pp. 235–240). An increase in content of this metal can be discerned (see Histogram I) from the embryo via the newborn and one to two weeks old animals to the three to 16 weeks old rats. It may be significant that the values for the regenerating livers are more like those of the earlier age groups, while those of the liver tumours are similar to the results with the later age groups.

Molybdenum (see Underwood[17], pp. 125–129). This trace element is of special interest to us in view of the work in these laboratories[9] on xanthine oxidase, a molybdeno-flavoprotein. The low levels of foetal and newborn livers rise over the next five weeks and arrive at an average value for the 16 weeks old, the pregnant females and the rats fed on kitchen scraps of 1·7 p.p.m. This should be contrasted with the content of the liver tumours of which three showed a low molybdenum concentration and four an average of 2·0 p.p.m. It has been mentioned before that the histological nature of the tumours was somewhat uncertain. This might explain the differences between the low and higher figures. The low values could be quoted in support of a report by Westerfeld and colleagues[8] on the diminished level of xanthine oxidase in livers of animals fed with butter yellow. Our figures obtained with foetal livers invite a similar comparison with the low level of xanthine oxidase activity in embryonic organs[34]. No molybdenum could be detected in our regenerating livers. If this is an observation reproducible with other material, it should be followed up by estimation of molybdeno-flavoproteins in regenerating organs.

Zinc (*cf.* Underwood[17], pp. 208–216). Our figures suggest a higher zinc content in the foetal, newborn and one week-old livers. Then they fall to an average of 102 p.p.m. in the organs of animals between 2 and 16-weeks old, organs with the data for all the other normal livers being about the same[35]. We cannot offer, at present, any explanation for the apparent absence of zinc in the regenerating livers. Some of the tumour livers did not yield results, because the presence of surprisingly high amounts of calcium made the spectrographic estimation of zinc impossible. This observation makes it very desirable to analyse at an early date for calcium. The remainder of the tumour-livers showed slightly higher metal contents, average 130 p.p.m. The other point which should be mentioned here, is that the keeping of rats in zinc cages has seemingly very little effect on the content of zinc in the livers.

There is no doubt that further work has to aim at the inclusion of other elements including major ones, such as sodium, potassium and calcium and to be backed up with coenzyme: enzyme determinations or assessment of other metal-bearing cellular constituents. Future studies should be extended to cellular particles and to other organs in the same species and to organs of other species, finishing in the end with comparable human material.

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