42. THE GLUTAMIC ACID OF NORMAL AND MALIGNANT TISSUE PROTEINS

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KÖGL & ERXLEBEN [1939, 1] have recently put forward the suggestion that partial racemization is a characteristic of the proteins of malignant tissues. The claim is based on their observation that a normal tissue such as calf lung, when submitted to a short acid hydrolysis, gave a series of amino-acids, all of which, after isolation, showed under standard conditions the normal rotation, whereas a few of the corresponding products from malignant tissues were found to be partially racemized. This difference was particularly striking in the case of glutamic acid. From normal lung and normal heart tissues they obtained, in what appeared to be good yields, the usual *l*-(+)-glutamic acid showing, in 9% hydrochloric acid, the normal rotation of $[\alpha]_D +31.6^\circ$. Corresponding preparations from an ovarian tumour, from an ovarian carcinoma and from two different mammary carcinomata, on the contrary, showed rotations of $[\alpha]_D +21.8^\circ$, $+4.6^\circ$, $+11.6^\circ$ and $+16.1^\circ$, indicating that they contained 15.6, 42.7, 31.7 and 24.6% respectively of *d*-(-)-glutamic acid.

The importance of these findings needs no emphasis, for they demonstrate a chemical differentiation of malignant and normal tissues which readily suggests several new paths of research into the origin and control of cancer. Confirmation in other laboratories was thus clearly desirable and as an extensive research had been in progress for some time at the Imperial College on the dicarboxylic acid content of various standard proteins the opportunity was taken to investigate the position with regard to glutamic acid.

The method of analysis which we employed in the first instance to isolate this acid as its hydrochloride was different from that of Kögl & Erxleben, and was essentially that of Foreman [1914], in which the aspartic and glutamic acids are first separated from the other amino-acid products of hydrolysis by precipitation as their calcium salts in the presence of alcohol. Applied to protein preparations from three tumours, viz. a Crocker sarcoma, a carcinoma of the bronchus and an extension in the neck from a carcinoma of bronchiole, we readily obtained in each case a first and large crop of glutamic acid hydrochloride which, on recrystallization from hydrochloric acid, had the normal rotation. The yields, calculated as glutamic acid in terms of weight of tissue preparation taken for analysis, varied between 5 and 7%, and as no attempt had been made to work up the mother liquors we knew from experience that these yields must be well below the maximum obtainable. Even so they compared favourably with all except two of those quoted by Kögl & Erxleben,¹ and we were accordingly led

¹ Kögl & Erxleben [1939, 1, 3] give many tables of tissue amino-acid analysis, in most of which appears the weight of glutamic acid obtained. It is clear however from the accompanying text that the weights quoted are really those of the hydrochlorides which they actually isolated, so that when referring to their yields of the free acid in the present paper it has been necessary for us to make the appropriate correction.

to suggest, in our preliminary communication [Chibnall *et al.* 1939], the need of a more extended investigation into these workers' claim.

Kögl & Erxleben [1939, 2, 3] have replied to this criticism, briefly in a letter to *Nature* and more extensively, with many new experimental data, in two papers (one with Akkerman [1939]), manuscript copies of which were very kindly sent to us before publication. *Inter alia* they produced evidence purporting to show that, under the modified Foreman conditions of analysis that we employed, the calcium salt of racemic glutamic acid has a much greater solubility than that of the natural antipode; furthermore they showed that the racemic hydrochloride has about twice the solubility of the corresponding natural product in either concentrated or 20 % hydrochloric acid at 0°, observations which led them to doubt whether our analytical procedure was suitable for demonstrating the presence of any partially racemized acid in our hydrolysates as our, admittedly large, crop of hydrochloride obtained by treatment of the calcium precipitate had been recrystallized from a relatively large volume of hydrochloric acid before the specific rotation had been determined. Indeed certain *ad hoc* experiments, in which samples of the racemic acid had been added to a hydrolysate of calf embryo tissue before analysis by the Foreman method provided them with what they regarded as conclusive evidence in support of their supposition, for the hydrochloride finally isolated had a rotation of $[\alpha]_D + 31.4^\circ$ characteristic of the natural *L*-(+)-glutamic acid. Finally, they reported further investigations of the partially racemized acid which they had previously obtained from a Brown-Pearce tumour and an ovarian carcinoma, leading in both cases to the isolation in pure form of the hydrochloride of the *d*-(−)-glutamic acid showing, in 9 % hydrochloric acid, a rotation of $[\alpha]_D - 31.2^\circ$.

This last finding constituted undeniable proof that the partially racemized acid had been present in these particular tumour hydrolysates, yet we were still puzzled to account for our own results quoted above. We had meanwhile examined the mother liquors from which our first crude crops of hydrochloride had been recrystallized and had been able to obtain evidence for only very small amounts of the *d*-(−) acid. The yield of the natural *L*-(+) acid had however been raised to nearly 10 %, and if, as Kögl & Erxleben maintained, we were employing a technique that failed to precipitate the racemic acid as the calcium salt, then the gross amount of glutamic acid in our tissue protein material must have been very much greater than that reported by these workers for the material which they themselves had analysed. It was necessary for us therefore to reinvestigate the problem, using these workers' analytical methods as well as our own.

In the first place we have been unable to confirm Kögl & Erxleben's observation that the calcium salt of the racemic acid has a much greater solubility than that of the natural antipode under the conditions of our modified Foreman analysis. As is shown in the experimental section, both products are equally insoluble. Proof that the racemic acid was indeed precipitated under these conditions was obtained by investigating the rotation of the successive fractions of glutamic acid hydrochloride which we had already obtained from a hydrolysate of wheat gliadin. In this analysis, which has not yet been taken to completion, the first main crop (fraction 1) was salted out with gaseous hydrogen chloride in the usual way and the product recrystallized before the application of our modified Foreman procedure to the collected mother liquors.

The necessary data are given in Table 1. It will be seen that the calcium salts have so far yielded fractions (2-6) representing 9.55 % of the protein as the natural *L*-(+) acid and 2.21 % as the *d*-(−) acid; in other words 46 % of the glutamic acid isolated *via* the Foreman procedure can be regarded as completely

Table 1. *Glutamic acid analysis of wheat gliadin*

Wt. of gliadin 49 g. N=17.8 %	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Yield % protein	
				<i>l</i> -(+) Glutamic acid	<i>d</i> -(-) Glutamic acid
	1	20.4	+31.4°	33.53	—
	2	4.322	+30.7°	7.13	—
	3	1.862	+ 1.0°	1.59	1.47
	4	0.671	+ 3.4°	0.61	0.49
	5 and 6	0.286	- 2.0°	0.22	0.25
	Total so far isolated			43.08	2.21

racemized. Similar results were obtained with edestin. Here the second and fourth fractions (the third was not available) had rotations of $[\alpha]_D + 23.4^\circ$ and $+ 7.3^\circ$ respectively, showing that at least 1.3 % of the protein was present in the hydrolysate as the *d*-(-) acid.

These results encouraged us to attempt further analysis of tumour tissue proteins to compare our own procedure with those employed by Kögl & Erxleben. Material prepared from metastatic deposits from the liver of a woman with carcinoma of the colon was analysed by the Foreman method and the results are given in Table 2. It will be seen that the last three fractions show partial racemization (we have adopted Kögl & Erxleben's limit of 30°), but the total amount of *d*-(-) acid present was very small, and more than a third of that suggested is due to fraction 2, a borderline case.

Table 2. *Glutamic acid analysis of protein material from metastases in the liver of a woman with carcinoma of the colon*

Wt. of material 34.5 g. N=16.2 %	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Yield % protein	
				<i>l</i> -(+) Glutamic acid	<i>d</i> -(-) Glutamic acid
	1	2.92	+31.6°	6.82	—
	2	1.34	+29.9°	3.012	0.088
	3	0.137	+13.2°	0.227	0.093
	4	0.11	+23.0°	0.225	0.035
	Total isolated			10.284	0.216

A companion analysis of 34.5 g. of material by the Dakin butyl alcohol method employed by Kögl & Erxleben when attempting to separate as many of the hydrolysis products as possible gave 3.11 g. of glutamic acid hydrochloride showing $[\alpha]_D + 29.0^\circ$ and again the amount of the *d*-(-) acid present was very small, about 0.15 % of the protein material. It may be mentioned here that the Foreman analysis ran parallel with those of the three tumour preparations (which could not be taken to completion because the final mother liquors had not been retained) referred to in our earlier communication: in all cases the major part of the glutamic acid was isolated in the first two crops, both of which showed, within reasonable limits, the normal rotation and there can be no doubt that the amounts of *d*-(-) acid in the hydrolysates are small.

We had thus been able to confirm the presence of partially racemized glutamic acid in tumour tissues, but in amounts far short of those claimed by Kögl & Erxleben, so small in fact that we doubted whether approximately the same yields could not be obtained from normal or non-malignant tissues. Using

the Dakin method Kögl & Erxleben had not been uniformly successful in their attempts to isolate glutamic acid hydrochloride from such tissue hydrolysates (see Table 4), and their evidence, the validity of which we discuss below, that it had the normal rotation, was based in part on products isolated by the cuprous oxide procedure of Abderhalden & Fuchs [1908]. Our analyses of normal heart and normal lung tissue proteins are given in Table 3 and it will be seen that the

Table 3. *Glutamic acid analyses of normal ox heart and normal calf lung tissue proteins*

	Fraction	Wt. of glutamic acid hydrochloride g.	[α] _D in 9% HCl	Yield % protein	
				<i>l</i> -(+) Glutamic acid	<i>d</i> -(-) Glutamic acid
Normal	1	4.22	+31.4°	7.20	—
ox heart	2	1.85	+31.2°	3.16	—
material	3	0.522	+30.4°	0.86	—
(47.2 g.)	4	0.359	+14.9°	0.71	0.16
N = 14.3%			Total isolated	11.93	0.16
Normal calf	1	2.70	+31.4°	7.80	—
lung material	2	0.12	+28.8°	0.335	0.015
(27.84 g.)	3	0.293	+26.5°	0.77	0.063
N = 15.3%			Total isolated	8.905	0.078

hydrolysates contained the *d*-(-) acid in amounts comparable with those which we had found in the tumour proteins. It is realized of course that in hydrolysates containing only very small amounts of the *d*-(-) acid the residual solubility of the calcium salt during the Foreman analysis, which cannot be foretold, may operate so as greatly to reduce the amount of the *d*-(-) acid actually isolated, but this will not affect the point at issue, which is the demonstration that the *d*-(-) acid is present in approximately equal amounts in the hydrolysates of both normal and malignant tissue proteins.

As the number of tumour tissues that we had so far examined was small we decided to investigate other samples by the cuprous oxide procedure, which Kögl & Erxleben had themselves used in the majority of cases for the isolation of the glutamic acid hydrochloride. From metastases in liver we readily obtained 5% of glutamic acid showing a rotation of [α]_D +30.5° and from grafted sarcomata of mice (Table 6) 5.0% showing a rotation of [α]_D +30.1°, products which again failed to substantiate these workers' claim.

This apparent impasse led us to examine more closely the data, summarized for convenience in Table 4, on which Kögl & Erxleben's claim was based. Analyses 1-8 were carried out by the Dakin method, and, as we have mentioned above, the yields of glutamic acid show very large variations. In analyses 3 and 5, however, both of malignant tissues, large yields were obtained, and, as they emphasize, the products exhibit a high degree of racemization, 89 and 80% respectively (cf. column 7). Using the cuprous oxide method the products isolated from malignant tissues also exhibited racemization in large though varying degree, whereas those from non-malignant tumours and normal tissues did not show this phenomenon. Viewed in this way the evidence for Kögl & Erxleben's claim that racemic glutamic acid is a characteristic of malignancy seems quite convincing. Yet underlying the claim is the assumption that the evidence provided by analyses 8, 9, 10, 11, 14, 23 and 25 is of equal weight to that of analyses 3 and 5. In accepting this, Kögl & Erxleben have relied on the

Table 4. *Glutamic acid analyses of normal and tumour proteins by Kögl & Erxleben*

No.	Material (1)	Wt. g. (2)	Method of analysis (3)	Reference* (4)	Total glutamic acid hydro- chloride isolated g. (5)	[α] _D in 9% HCl (6)	Percentage of isolated glutamic acid in d-(-) form (7)	Yield % protein	
								Total glutamic acid (8)	d-(-) Glutamic acid (9)
1	Normal calf lung	15	Dakin	A, Table 1	None isolated	—	—	—	—
2	Rabbit tumour	12.5	"	A, Table 2	None isolated	—	—	—	—
3	Rabbit tumour	18	"	A, Table 4	1.85	+ 3.5°	44.5	8.27	3.69
4	Mouse adenocarcinoma	17.7	"	A, Table 3	5.61	—	—	—	—
5	Human ovarian carcinoma	48.3	"	A, Table 5	0.395	+ 3.2°	40.0	9.49	4.16
6	Uterine myoma	85.0	"	C, Table 1	None	+ 30°	—	0.37	—
7	Rabbit myoma	38.2	"	C	0.092	—	—	—	—
8	Rabbit myxoma	18.7	"	A, Table 6	1.05	+ 20.5°	15.0	0.4	0.06
9	Ovarian tumour	99.5	Cuprous oxide	"	0.205	+ 21.8°	15.6	0.85	0.13
10	Mammary carcinoma	50.9	"	"	0.156	+ 11.6°	31.7	0.32	0.10
11	Mammary carcinoma	20.6	"	"	0.810	+ 16.1°	24.6	1.65	0.15
12	Normal ovary	39.6	"	"	0.104	+ 31.6°	—	—	—
13	Normal ovary	2.62	"	"	0.475	+ 31.5°	—	3.20	—
14	Omentum tumour of rabbit	13.66	"	"	0.16	+ 4.6°	42.6	2.80	1.20
15	Normal heart from above	3.3	"	"	0.194	+ 31.1°	—	3.90	—
16	Normal lung from above	4.1	"	"	0.136	+ 31.4°	—	3.51	—
17	Normal rabbit heart	3.7	"	"	0.316	+ 31.3°	—	2.96	—
18	Normal rabbit lung	5.1	"	"	0.346	+ 31.4°	—	4.99	—
19	Calf embryo	10	"	B	1.387	+ 31.5°	—	2.79	—
20	Calf embryo	15.3	Foreman	B	0.595	+ 28.8°	—	7.30	—
21	Human myoma	17.6	Cuprous oxide	C	0.54	+ 29.7°	—	2.72	—
22	Human myoma	17.8	"	C	0.611	+ 21.2°	15.1	2.40	—
23	Human carcinoma, metastases from spleen	13.8	"	C	0.475	—	—	3.57	0.53
24	Human lung with secondary carcinoma: (1) Normal part	9	"	C	0.395	+ 30.2°	—	4.25	—
25	(2) Tumour part	6.5	"	C	—	+ 24.4°	10.1	4.87	0.54

* A, Kögl & Erxleben [1939, 1]; B, Kögl *et al.* [1939]; C, Kögl & Erxleben [1939, 3].

data given in column 7 and have not considered at all the data we have calculated and give in columns 8 and 9. As protein chemists our attention has throughout been focused on the *yields* of glutamic acid which we could isolate from both malignant and normal tissue protein preparations and when we have attempted to make the separation as quantitative as possible the yields have been uniformly high. Now the cuprous oxide procedure of Abderhalden & Fuchs [1908], which is merely a minor modification of the very early method of salting out the glutamic acid as its hydrochloride from the complete mixture of hydrolysis products by saturation with hydrogen chloride, cannot be quantitative (cf. Tables 5 and 6). It appeared to us therefore that the evidence provided, for instance, by analyses 9, 10 and 11, if interpreted in terms of the data given in column 7, would not provide a valid comparison with the corresponding data from analyses 3 and 5 unless it could be shown that the glutamic acid isolated in such *very small* yields in the former cases was representative of the total amount of this acid present in the hydrolysate, i.e. that it contained at least an average amount of the *d*-(-) antipode. Kögl & Erxleben have assumed that this must be so on the strength of Akkerman's observation that the racemic hydrochloride had a greater solubility in hydrochloric acid than the natural product, which would indicate that the residual acid might be even more completely racemized than the product actually isolated. In our Foreman analyses this relationship undoubtedly held, for the small amount of partially racemized acid was obtained from the later, more soluble, fractions. We remembered however that the material removed as insoluble calcium salt from the hydrolysis mixture by this method consisted essentially of aspartic and glutamic acids and that the major part of the former acid was separated as its copper salt early in the subsequent fractionation; consequently the fact that Akkerman's relationship governed the isolation of glutamic acid hydrochloride from the later mother liquors was to be expected. Yet we were not sure that this would necessarily be so in the cuprous oxide analyses, wherein the glutamic acid hydrochloride was salted out from a very complex mixture of amino-acid hydrochlorides. We had, without difficulty, obtained glutamic acid in yields of 5% by this method and the product had shown no appreciable racemization, yet Kögl & Erxleben, in their analysis 10 (Table 4) had obtained a product showing very complete racemization in a yield as low as 0.32%. Such a yield, moreover, represented only 0.1% of the *d*-(-) antipode, and was thus of the same order as we ourselves had found ultimately in both normal and tumour tissue proteins. This suggested to us the possibility that under the conditions of their cuprous oxide analyses there might have been a preferential salting out of the racemic hydrochloride in the presence of much larger amounts of the natural antipode.

Appropriate experiments showed that this was indeed the case. The conditions of analysis prescribed by Kögl & Erxleben were followed exactly, care being taken to seed the solution with a small crystal of the racemic hydrochloride. In the first experiment, using material from metastases in liver, three successive crops were removed and recrystallized, and as will be seen from the data given in Table 5 it is only the first of these which shows evidence of partial racemization. As this crop represented nearly 1% of the protein material taken for analysis all three crops were returned to the mother liquor and the isolation repeated. We were successful this time in obtaining a first crop, representing only 0.44% of the material, which showed a high degree of racemization. Conditions were then adjusted so as to obtain as large a second crop as possible, in order to permit comparison with our earlier results by this method. The total yield of glutamic acid was 2.83%, very much less than that given by the companion Foreman

Table 5. *Glutamic acid analysis of protein material from metastases in liver of a man with carcinoma of the bronchus*

Method of analysis	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Percentage of isolated glutamic acid in <i>d</i> -(-) form	Yield % protein	
					Total glutamic acid	<i>d</i> -(-) Glutamic acid
Cuprous oxide	1	0.218	20.8°	17	0.93	0.16
19 g.: N=15.5%	2	0.16	29.0°	4	0.66	0.03
1st analysis	3	0.27	31.0°	—	1.15	—
Total isolated		0.648	—	—	2.74	0.19
Same material:	1	0.103	11.6°	31.7	0.44	0.14
2nd analysis	2	0.565	31.0°	—	2.39	—
Total isolated		0.668	—	—	2.83	0.14
Foreman 39 g.	1	3.88	31.4°	—	8.04	—
	2	0.572	26.5°	8	1.18	0.074
	3	0.326	29.5°	3.5	0.67	0.023
	4	0.178	19.8°	19	0.36	0.068
Total isolated		4.956	—	—	10.25	0.165

analysis. Analogous results were given by protein material from grafted sarcomata of mice (Table 6), although in this case the degree of racemization observed in the first crop of glutamic acid hydrochloride was not so pronounced. Likewise with normal heart muscle the rotation of the first small crop of hydrochloride was fairly high $[\alpha]_D + 29^\circ$. These variations may be merely reflecting the small amount of the *d*-(-) antipode in the hydrolysate concerned; on the other hand it must be remembered that the solubility of the racemic hydrochloride, like that of any other amino-acid or its salt, must be a function of the dielectric constant of the medium [cf. Cohn, 1935], and in the case of protein hydrolysates this will depend on the particular amino-acid mixture present therein, a point that may be lost sight of by workers inexperienced in protein analysis.

Table 6. *Glutamic acid analysis of protein material from grafted sarcomata of mice*

Method of analysis	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Percentage of isolated glutamic acid in <i>d</i> -(-) form	Yield % protein	
					Total glutamic acid	<i>d</i> -(-) Glutamic acid
30 g.: N=15.0%						
1st analysis by cuprous oxide	1	1.85	+30.3°	—	4.97	—
Repeat analysis of same material	1	0.1468	+28.3°	5.4	0.39	0.02
	2	0.3988	+31.0°	—	1.07	—
Foreman analysis on mother liquor of above	3	2.24	+31.4°	—	6.01	—
	4	0.323	+24.8°	9	0.87	0.094
Total isolated		3.1086	—	—	8.34	0.114

The results given in Table 5 are most instructive. Fraction 1 of the second cuprous oxide analysis is very similar to the analysis 10 (Table 5) of Kögl & Erxleben mentioned above; both samples of glutamic acid hydrochloride show the same high percentage racemization (63.4%), yet the other data given in Table 5 leave no doubt that fraction 1 is in no way representative of the re-

maintaining glutamic acid of the hydrolysate. Indeed the maximum amount of the *d*-(-) antipode present in the whole hydrolysate was no more than about 0.2% of the protein material, so that the percentage racemization of the total glutamic acid was no more than 4%. Such, admittedly somewhat unexpected, findings provide a complete explanation of the many anomalous results obtained by other workers besides ourselves [cf. White & White, 1939; Arnow & Opsahl, 1939; Graff, 1939]. In those cases in which the dicarboxylic acids have been separated by Foreman's method the first two or three crops of glutamic acid hydrochloride will show very little, if any, racemization if the natural antipode be present in large excess, whereas the product salted out from the complete hydrolysis mixture of amino-acids (cuprous oxide) or an attenuated mixture (Dakin), provided the yield be small enough, may exhibit considerable racemization. Similar reasoning also explains the results of Kögl, Erxleben & Akkerman's experiments in which the racemic acid had been added to the hydrolysate of normal calf embryo, for the single-step Foreman analysis which they performed has undoubtedly failed to reveal the maximum amount of the natural antipode originally present therein.

Reverting finally to Kögl & Erxleben's claim that partially racemized glutamic acid is a characteristic of malignancy we see at once that the interpretation by them of the data given in Table 4 in terms of percentage racemization of the isolated product (column 7) is not a valid procedure. If comparison of their cuprous oxide analyses with our own can be taken as a guide, then, in the absence of definite evidence to the contrary, we must assume that their yields of the *d*-(-) antipode (column 9), which show wide variations as between one tumour and another, will not be far short of the total amounts present in the respective hydrolysates. The yields in two cases (analyses 3 and 5) are indeed extremely large, while in another (analysis 14) the yield is larger than we have encountered, but in the remainder it is of the same order of magnitude as we ourselves have found, not only in hydrolysates of seven different samples of tumour protein material but also, if appropriate analytical methods be employed, in those of two normal animal proteins as well. The sharp differentiation which Kögl & Erxleben thought they had established as between normal and non-malignant tissue proteins on the one hand and malignant tissue proteins on the other hand is thus no longer valid and since the grafted sarcomata of mice which we examined (Table 6) consisted almost wholly of malignant tissue there can be no doubt that the occurrence of *d*-(-)glutamic acid is not in any way a characteristic of malignancy.

The early masters of protein analysis, Emil Fischer and T. B. Osborne, always advised against the comparison of proteins on a basis of amino-acid composition, for protein analyses were then, as they remain in most cases to-day, far from being quantitatively complete. This criticism can be applied to Kögl & Erxleben's more extensive analyses of certain of their samples of normal and tumour protein material, for although most of the known protein amino-acids have been isolated, the overall yields represent, even in the most favourable cases, no more than half the protein material taken for analysis. Moreover, they were not comparing one particular protein with another; for both normal and malignant tissues contain a mixture of these substances, the individual members of which will be different in each particular normal or malignant tissue concerned. Consequently their various tissue hydrolysates may have contained very different amino-acid mixtures, a position which might profoundly affect the yield of an amino-acid separated by a method involving the solubility of derivatives such as copper salts. Copper aspartate, for instance,

is insoluble in cold and nearly insoluble in boiling water, yet during our analysis of various pure proteins for dicarboxylic acids we have frequently obtained fractions of copper salts freely soluble in cold water from which we have subsequently isolated, after appropriate treatment, 30–40 % of copper aspartate. Such experience leads us to doubt whether any importance can be attached to the isolation by Kögl & Erxleben from samples of malignant tissue protein material of, for instance, leucine and valine exhibiting a few per cent racemization until evidence is produced to show that the yields in these and in the comparative analyses of normal tissue material are well nigh quantitative, for the conditions governing the solubilities of the lead and copper salts of the racemized acids during the fractionation of the hydrolysis products may have been different in the two cases, thus masking their presence in the normal tissue.

Table 7. *Aspartic acid analyses of normal and malignant tissue protein material*

Material	Fraction	Wt. of copper salt g.	[α] _D in 10 % HCl	Yield % protein	
				Total aspartic acid	<i>d</i> -(+) Aspartic acid
Normal heart muscle (47.2 g.) (compare Table 3)	1	4.533	+25.6°	4.64	—
	2	0.59	+24.0°	0.90	0.03
	3	0.29	+ 0.7°	0.58	0.29
	4	0.566	—	6.12	0.32
Total isolated		5.979	—	6.12	0.32
Metastases from liver (34.5 g.) (compare Table 2)	1	3.98	+25.7°	5.57	—
	2	0.64	+24.6°	0.91	—
	3	0.346	+ 4.5°	0.50	0.20
	Total isolated	4.966	—	6.98	0.20

An interesting illustration of this is afforded by a comparison of Kögl & Erxleben's and our own analyses for aspartic acid. The former workers' yields from both malignant and normal tissue materials were low and the products isolated showed no racemization; our own yields, on the contrary, we believe to be wellnigh quantitative (Table 7) and although the first and largest fraction in each case showed no racemization there was clear evidence of this in the later ones. We have thus another instance of non-correlation between racemization and malignancy.

Kögl & Erxleben admit that the "unnatural" *d*-amino-acids might be constituents of the protein molecule of a normal tissue and emphasize that their occurrence must not be regarded *a priori* as "pathological". But in these cases they suggest that the *d*-amino-acids are to be regarded as being built into the molecule in a "sensible" (*sinnvolle*) way as opposed to the "senseless" (*sinnlose*) way in which they become incorporated in the protein molecule of cancer tissues. By this they mean, we presume, that the 2.2 % of *d*-(−)glutamic acid we find in wheat gliadin and the 0.16 % we find in normal heart tissue protein are to be regarded as "normal constituents" of the proteins concerned, whereas the 4.0 % they have found in two malignant tissue proteins are evidence of some "disorder" in the protein metabolism. The data given in the present paper show that such speculations are as yet uncalled for, since the high yields in the latter cases appear to be themselves abnormal for the tissues concerned. Protein chemists have so far given but little attention to the occurrence of racemized amino-acids in protein hydrolysates and it may well be that they are more widespread

than has been imagined, for the possibility that racemization accompanies the splitting of certain peptide bonds during hydrolysis of the protein molecule must not be lost sight of.

EXPERIMENTAL

As we pointed out in our earlier communication [Chibnall *et al.* 1939], the hydrolysis procedure of Kögl & Erxleben (7 hr. with conc. HCl) does not effect the maximum splitting of peptide bonds and in our modified Foreman analyses quoted below we have preferred the more usual hydrolysis with 20 % HCl for 20–24 hr. Appropriate experiments have shown that when pure *l*-(+)-glutamic acid is submitted to such hydrolysis and then passed through the complete Foreman procedure no appreciable racemization occurs. The crystalline hydrochloride recovered represented 96.1 % of the original glutamic acid.

In the analyses described below the purity of the various crops of recrystallized glutamic acid hydrochloride was controlled by determining total N, and by confirming that the titration value in the micro-Kjeldahl was exactly one-half of that obtained when the same weight of material in aqueous solution was titrated to pH 7.3 (blue tint with bromothymol blue). To avoid repetition in the text it may be stated that all rotations of glutamic acid hydrochloride were observed in 9 % HCl and calculated as glutamic acid.

Solubility of racemic calcium glutamate under the conditions of a modified Foreman analysis. Kögl, Erxleben & Akkerman's [1939] procedure was followed exactly, although the aqueous volume from which the Ca salts were precipitated was about one-tenth of that normally used in our protein analysis. A mixture of 756.8 mg. of *l*-(+)-glutamic acid and 824.3 mg. of synthetic racemic glutamic acid (+ H₂O), giving, by calculation, a rotation of $[\alpha]_D + 15.6^\circ$, were dissolved in 55 ml. of warm water and freshly prepared calcium hydroxide cream was added in excess. The solution was filtered, the residue washed with warm water and the collected filtrate reduced *in vacuo* to 6.5 ml. when a small amount of Ca salt separated. Without removing this material 60 ml. of 95 % alcohol were added, the precipitated Ca salts filtered off on a sintered glass crucible and washed with a few ml. of absolute alcohol. The Ca salts were redissolved with difficulty, about 150 ml. of boiling water being required. The small insoluble residue, presumably mostly CaCO₃ derived from the excess Ca(OH)₂, precipitated from the original aqueous solution by the alcohol, was filtered off; analysis showed that this contained only 0.138 mg. N. The aqueous solution was concentrated as before and the precipitation repeated. Freed as it was from a small excess of lime the precipitate was this time very insoluble, and 600 ml. of boiling water were required to redissolve it. A small residue was filtered off (N = 0.4 mg.) and Ca removed from the filtrate while this was still warm. The precipitated Ca oxalate was washed four times by boiling with portions of 150 ml. of water and centrifuging, the collected liquors reduced *in vacuo* at 40° to about 150 ml., the final adjustment for the removal of Ca made with oxalic acid, and the filtered solution concentrated *in vacuo* to 70 ml. A few ml. of HCl were then added and the solution taken finally to dryness in a vacuum desiccator. The glutamic acid hydrochloride recovered weighed 1.872 g. and it showed a rotation of $[\alpha]_D + 15.6^\circ$. When the material was dissolved in water there remained a small residue, about 2 %, of Ca oxalate; this was filtered off, the volume adjusted (warm) to 10 ml. of 20 % HCl and the solution allowed to crystallize at room temperature. 1.37 g. of hydrochloride were recovered; $[\alpha]_D + 19.8^\circ$. The two aqueous-alcoholic filtrates were taken to dryness and submitted to Kjeldahl analysis: 1.57 mg. of N were found, corresponding to just over 1 % of that present in the original mixture of

glutamic acids. The large loss encountered by Kögl & Erxleben cannot therefore be attributed to the solubility of either the racemized or the *d*-(-) antipode Ca salt and it must have been due to incomplete re-solution of the Ca salts or to crystallization of these from solution during removal of Ca if this operation had been performed in the cold. It may be remarked that in a protein analysis by our normal procedure we have encountered no difficulty in redissolving the Ca precipitates, owing no doubt to the presence of the CaCl_2 arising from the combined HCl of the amino-acid hydrochlorides in the hydrolysates.

Protein material from normal ox heart

Fresh ox heart was washed carefully to remove blood and finely minced. 1200 g. of the minced material were extracted twice with 3 l. of ice-cold 5 % trichloroacetic acid and then washed three times with 2 l. of acetone. The residue was dried at 50° and then extracted thoroughly, first with boiling 95 % alcohol and finally with ether.

Foreman analysis. To illustrate the intricacies of the modified Foreman procedure we have used in the present research we shall describe this ox heart protein analysis in full. Brief details only will be given of the remaining analyses, wherein minor modifications were occasionally introduced as circumstances warranted.

47.2 g. of dry material were hydrolysed with 6 vol. of 20 % HCl for 22 hr. The excess HCl was then removed in the usual way by repeated evaporation to a syrup *in vacuo*, cream of lime added and the excess lime, together with the humin, removed by centrifuging. The lime-humin residue was washed four times with 150 ml. of hot water and the collected liquors evaporated *in vacuo* to remove ammonia. The volume was then made up to 400 ml. and 3420 ml. of absolute alcohol added with vigorous stirring. The separated Ca salts were filtered off without delay on a Büchner funnel and the extraneous mother liquor removed by pressing the mass to a hard pad, which was then washed with a small volume of absolute alcohol. On redissolving the material in about 300 ml. of warm water a small residue, presumably CaCO_3 , remained undissolved. Without removing this, 10 ml. of cream of lime were added, the volume adjusted to 400 ml. and the precipitation with alcohol repeated. The washed Ca salts were dissolved out with 800 ml. of warm water, the small residue washed four times by stirring with 150 ml. of water kept near the boil for a few minutes, centrifuged and the clear liquors collected. Ca was removed with oxalic acid without delay to prevent crystallization of the relatively insoluble Ca salts; the Ca oxalate was washed four times with 150 ml. of hot water as described above, the mixture cooled to room temperature and kept for some time to permit recrystallization of the small amounts of oxalate redissolved. The oxalate was then removed by centrifuging, the clear liquors collected and reduced *in vacuo* to about 80 ml. Final adjustment to remove residual Ca or oxalic acid was then made, the precipitate centrifuged off and washed as before, and about 20 ml. of HCl added to the clear liquors which were then evaporated to a syrup. On standing overnight at room temperature much glutamic acid hydrochloride crystallized out. This was removed, redissolved in about 20 ml. of water, 5 ml. of HCl were added and the mixture concentrated in a dish on the water bath to incipient crystallization. Next day the material was filtered off on a sintered glass filter, washed with HCl and dried over NaOH in a vacuum desiccator. This first crop of glutamic acid hydrochloride weighed 4.22 g. and the rotation was $[\alpha]_D + 31.4^\circ$ (fraction 1, Table 3). By treatment of the mother liquors a

second crop of hydrochloride was obtained, which was recrystallized as before. The weight was 1.852 g. and the rotation $[\alpha]_D + 31.2^\circ$ (fraction 2, Table 3).

The mother liquors were again collected, excess HCl removed by repeated evaporation *in vacuo* to a syrup, which was then taken up in 500 ml. of water, boiled with sulphate-free CuCO_3 , the excess removed by filtration on a hot Büchner funnel and the filtrate and washing reduced *in vacuo* to 170 ml. On standing at room temperature overnight a large amount of greenish-blue, and therefore impure, Cu aspartate separated. This was filtered off, suspended in 500 ml. of water kept near the boil, HCl added drop by drop until solution was complete, CuCO_3 added and the excess removed by filtration on a hot Büchner funnel. The clear filtrate was kept overnight, when 4.533 g. of pure (blue) crystalline Cu aspartate separated.¹

Cu was removed from the first Cu filtrate with H_2S , excess HCl removed by evaporation in the usual way and a third Foreman precipitation of the Ca salts made from an aqueous volume of 100 ml. On treatment as before there was obtained a third crop of glutamic acid hydrochloride weighing 0.7498 g. Recrystallized from 20 % HCl this gave a crop of 0.654 g., the mother liquor being reserved for treatment as described below. A small amount of difficultly soluble inorganic material was removed from this crop by repeated solution in a small volume of water and filtration, when there was finally obtained 0.5222 g. of hydrochloride with a rotation of $[\alpha]_D + 30.4^\circ$ (fraction 3, Table 3).

The Cu treatment was next applied to the main mother liquor when two crops (0.59, 0.29 g.) of pure recrystallized Cu aspartate were obtained. Following this, Cu was removed in the usual way and a fourth crop of glutamic acid hydrochloride (0.1762 g.) obtained. The filtrate was then again passed through the Foreman procedure (aqueous volume 25 ml.) to give a fifth crop (0.2836 g.) of hydrochloride. The filtrate from this was given another Foreman precipitation (aqueous volume 20 ml.) and the solution of free amino-acids recovered from the Ca salts evaporated to a small volume *in vacuo*. On standing 0.1232 g. of crude tyrosine separated. The mother liquor was then concentrated slowly to a syrup in a vacuum desiccator and after 2 days a few drops of alcohol were stirred in. On standing, 0.78 g. of material separated; from 0.4 g. of this a fourth crop of Cu aspartate (0.27 g.), but no glutamic acid, was separated. The ultimate mother liquor, on treatment with HCl, finally gave a sixth crop (23 mg.) of glutamic acid hydrochloride. Crops 4, 5 and 6, with the mother liquor from the recrystallization of crop 3, were collected, dissolved in about 2–3 ml. of cold water, filtered to remove an insoluble inorganic residue and the filtrate reduced to dryness *in vacuo*. This treatment was repeated until a clear solution was obtained without filtration. On crystallization from 20 % HCl there was then obtained 0.358 g. of glutamic acid hydrochloride (fraction 4, Table 3) with a rotation of $[\alpha]_D + 14.9^\circ$. It may be mentioned here that contamination of the later fractions of the hydrochloride with inorganic material was always observed in the case of the crude tissue hydrolysates discussed in the present paper, but we have not so far encountered it with corresponding fractions from pure proteins. As will be seen from Table 3, the gross yield of glutamic acid was 11.93 % and of the *d*-(–) antipode 0.16 %; 2.6 % of the glutamic acid can therefore be regarded as fully racemized.

Aspartic acid was recovered from the Cu salt by dissolving in the necessary volume of boiling water and removing Cu with H_2S . The recovery of crystalline

¹ The purification of these crude Cu salts needs experience, as the impurities present may separate either before or after the Cu aspartate during recrystallization. We shall refer to this more fully in a later paper.

acid was between 95 and 100 %. The product obtained from the first crop of Cu salt had the normal rotation of the *l*-(-) antipode in 10 % HCl of $[\alpha]_D + 25.6^\circ$; that from the second and third crops combined had $[\alpha]_D + 24^\circ$, while that from the fourth crop showed $[\alpha]_D + 0.7^\circ$. The overall yield of aspartic acid was 6.12 % and of *d*-(+) antipode 0.32 %; 10.5 % of the total acid can therefore be regarded as fully racemized (Table 7).

Abderhalden & Fuchs (cuprous oxide) analysis. Kögl & Erxleben's procedure, certain points of which are emphasized in the first malignant tumour analysis described below, was followed. From 28.3 g. of protein material a first recrystallized crop (0.12 g.) of glutamic acid hydrochloride was obtained which showed $[\alpha]_D + 29^\circ$. Repetition of the procedure several times did not give a first crop showing any higher degree of racemization.

Protein material from normal calf lung

Fresh calf lung (500 g.) was finely minced and extracted with 1500 ml. of ice-cold 5 % trichloroacetic acid. The material was again treated in the same way and then washed three times with 1 l. of acetone. The residue was dried at 50° and then thoroughly extracted, first with boiling 95 % alcohol and then with ether.

27.8 g. of dry material were hydrolysed with 6 vol. of 20 % HCl for 22 hr., and then passed through the Foreman procedure detailed above. Data are given in Table 3. After the initial double precipitation (aqueous volume 200 ml.) 3.255 g. of crude hydrochloride were separated; on recrystallization this gave two crops, 2.70 and 0.12 g., showing $[\alpha]_D + 31.4^\circ$ and $+ 28.5^\circ$ respectively (fractions 1 and 2, Table 3). A first crop of crude Cu aspartate was then removed; on recrystallization this gave 2.26 g. of pure Cu aspartate. A third Foreman precipitation (aqueous volume 25 ml.) gave a third crop (0.288 g.) of glutamic acid hydrochloride, but no insoluble Cu salt could be obtained. A fourth Foreman process (aqueous volume 15 ml.) gave a fourth crop (70 mg.) of hydrochloride and a small second crop (0.4 g.) of recrystallized Cu aspartate. The mother liquor, on standing at room temperature for 14 days, slowly deposited a third crop (0.17 g.) of Cu aspartate. The third and fourth crops of hydrochloride were combined with a small crop (37 mg.) obtained from the mother liquor of crop one; inorganic material was removed as before and 0.293 g. of material showing $[\alpha]_D + 26.5^\circ$ obtained (fraction 3, Table 3). The overall yield of glutamic acid was 8.9 %, of the *d*-(-) antipode 0.078 % and of aspartic acid (not further characterized) 4.92 %; 1.8 % of the total glutamic acid isolated can thus be regarded as completely racemized.

*Protein material from metastases in liver of a man with carcinoma
of the bronchus*

Malignant nodules from the liver of a man (W. V. aged 55 years) who had died from carcinoma of the bronchus were separated from the liver tissue. The material (2500 g.) was minced and extracted in the cold with 10 l. of 0.6 % NaCl. 4 vol. of 90 % alcohol were added to the separated extract, and the precipitated protein dried at 50° (108 g.). The residue after extraction was washed with 80 % alcohol and dried at 100° (260 g.). The two products were combined, finely ground, washed with warm water until free from NaCl, then repeatedly with boiling 95 % alcohol and finally with ether.

Abderhalden & Fuchs (cuprous oxide) analysis. 19 g. of dry material were hydrolysed for 7 hr. with three times the weight of HCl (sp. gr. 1.19) and then treated exactly as described by Kögl & Erxleben [1939, 1]. The pale yellow

hydrolysate thus obtained was concentrated to a thick syrup (20 ml.) in a 50 ml. centrifuge tube, seeded with a small crystal of the racemic hydrochloride and kept in the ice box at 0°. During the course of 2 days a mass of large crystals appeared around the nucleus in the upper layer of the syrup, together with a much finer white crystalline flocculum which was dispersed throughout the remainder of the syrup. The separated solid was then filtered off on a sintered glass filter, washed with a few drops of HCl and dried *in vacuo* over NaOH. The material (0.2927 g.; $[\alpha]_D + 20.4^\circ$) was recrystallized from 1 ml. of 20% HCl (0.2183 g.; $[\alpha]_D + 20.8^\circ$). The collected mother liquors were treated twice successively in the same way, and further crops of recrystallized material obtained. (Data given in Table 5, 1st analysis.)

To obtain, if possible, a first crop showing more complete racemization, all the materials of this first analysis were collected, returned to the mother liquor and the treatment repeated. As before, large crystals formed around the nucleus in the upper layer of syrup and a white flocculum appeared throughout the rest of the liquid. On centrifuging, these crystals remained at the top, while the flocculum collected at the bottom of the clarified liquid, and it was possible to filter off the former without contamination with the latter. The crop weighed 0.1274 g. and showed $[\alpha]_D + 9.5^\circ$. Recrystallized from 1 ml. of 20% HCl this gave 0.103 g. showing $[\alpha]_D + 11.6^\circ$. The mother liquors were collected, treated as before, and left for 2 days at 0°. The recrystallized material weighed 0.564 g. and showed $[\alpha]_D + 31.0^\circ$ (see Table 5, 2nd analysis).

Foreman analysis. The conditions of this analysis followed closely those described above for normal lung tissue material, and the data for the four fractions of glutamic acid hydrochloride obtained are given in Table 5; 2.8% of the total glutamic acid isolated can be regarded as completely racemized. Two crops of recrystallized Cu aspartate were obtained (5.22 g. and 0.18 g.), and the yield of aspartic acid (not further characterized) was 6.69%.

*Protein material from metastases in liver of a woman with
carcinoma of the colon*

Metastatic deposits (750 g.) from the liver of a woman (E. B. aged 73 years) with carcinoma of the colon were minced and extracted with 3 vol. of 3% trichloroacetic acid. The insoluble material was washed once with the same reagent, then three times with acetone and dried. The material was finely ground, exhaustively extracted with boiling 95% alcohol and then with ether.

Dakin (butyl alcohol) analysis. 34.5 g. of dry material were hydrolysed for 7 hr. with three times the weight of HCl (sp. gr. 1.17) and passed through the procedure outlined by Kögl & Erxleben [1939, 1]. Three successive crops of crude hydrochloride were separated and the collected material recrystallized from 20% HCl. 3.11 g. of glutamic acid hydrochloride were thus obtained, showing $[\alpha]_D + 29.0^\circ$. The yield of glutamic acid (7.26% and at the most only 0.2% of the *d*-(-) antipode) was lower than that of the companion Foreman analysis given below, owing undoubtedly to incomplete hydrolysis, and perhaps in part to the conditions under which the monoamino-acids were extracted not being optimal, leading to loss of dicarboxylic acids from the aqueous solution [cf. Sharp, 1939].

Foreman analysis. 34.5 g. of dry material were hydrolysed for 20 hr. with 6 vol. of 20% HCl. The analysis followed closely those described above for normal heart material, and the data for the glutamic acid isolated are given in Table 3. It will be seen that 4.3% of the isolated glutamic acid can be regarded

as completely racemized. After the second and third Foreman precipitations crops of recrystallized Cu aspartate were removed. Following the fourth precipitation a crystalline crop (0.4738 g.) of amino-acids was obtained by evaporation of the Ca-free filtrate; from this 0.346 g. of Cu aspartate was separated. Data for the aspartic acid recovered from the three crops of salt are collected in Table 7. It will be seen that the yield of aspartic acid was 6.98% and of the *d*-(+) antipode 0.20%, so that 5.6% of the acid can be regarded as completely racemized.

Protein material from grafted sarcomata of mice

Grafted sarcomata of the strain M.C.D.B. 1 (see Boyland & Boyland [1939]) were removed from mice and finely minced. The material (240 g.) was extracted twice with 3% trichloroacetic acid and the residue washed twice with acetone and dried. The product was finely ground and exhaustively extracted first with boiling 95% alcohol and then with ether.

30 g. of dry material were hydrolysed with three times the weight of HCl (sp. gr. 1.17) for 7 hr. and the cuprous oxide procedure applied. In the first separation 1.85 g. of recrystallized glutamic acid hydrochloride were obtained, showing $[\alpha]_D + 30.3^\circ$ (Table 6, 1st analysis). The material was returned to the mother liquor and conditions adjusted (as described above) to yield two small successive crops. On recrystallization these weighed 0.1482 and 0.3988 g., showing $[\alpha]_D + 28.3^\circ$ and $+31.0^\circ$ respectively (Table 6, 2nd analysis). The collected mother liquors were then given a double Foreman precipitation (aqueous volume 250 ml.) and two crops (2.05, 0.296 g.) of crude hydrochloride removed. These were combined and recrystallized from 20% HCl to give 2.24 g. of glutamic acid hydrochloride: $[\alpha]_D + 31.4^\circ$ (Table 6, fraction 3); 2.29 g. of Cu aspartate were next obtained, followed by a third crop (0.2 g.) of crude hydrochloride. Since much intractable material remained in the mother liquors at this stage they were hydrolysed with 20% HCl for 20 hr., followed by a single Foreman precipitation (aqueous volume 20 ml.). A fourth crop of crude hydrochloride (0.192 g.) was then removed, next 0.763 g. of Cu aspartate and finally a fifth crop (38 mg.) of crude hydrochloride. Crops 3, 4 and 5 were collected, insoluble inorganic material removed as described before, and the material recrystallized from 20% HCl; 0.323 g. of glutamic acid hydrochloride was thus obtained, showing $[\alpha]_D + 24.8^\circ$ (Table 6, fraction 4). The overall yield of glutamic acid was 8.34%, of the *d*-(-) antipode 0.114%, and of aspartic acid 4.96%: all of them will be less than the maximum obtainable as complete hydrolysis was not attained until much material had been rejected by the Foreman treatment.

SUMMARY

The claim of Kögl & Erxleben that racemization of amino-acids, especially of glutamic acid, is a characteristic of the proteins of malignant tissues has been investigated in some detail.

Quantitative isolation of glutamic acid by a modification of Foreman's method from seven different malignant tissue protein preparations showed that in all cases the yield was high (*ca.* 10%) and that the degree of racemization was small, the yield of the *d*-(-) antipode being only about 0.2%. Similar results were obtained from two normal tissue protein preparations; gliadin, a plant protein, gave 2.2% of the *d*-(-) antipode.

The cuprous oxide (Abderhalden & Fuchs) method used by Kögl & Erxleben to isolate glutamic acid as its hydrochloride from tissue hydrolysate has been shown to give the racemized product preferentially, even when a large excess

of the *l*-(+) antipode is present. The interpretation by Kögl & Erxleben of their results obtained by this method is thus shown to be invalid.

It is shown that small amounts of partially racemized aspartic acid, as well as of glutamic acid, can be isolated from both normal and malignant tissue protein material and it is concluded that racemization of amino-acids is not a characteristic of the proteins of malignancy.

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