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On Ergothioneine in Blood and Diazo-reacting Substances in Maize

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(Received 22 July 1950)

Ergothioneine, discovered in ergot of rye (Tanret, 1909), was isolated from pig blood by Hunter & Eagles (1925, 1927) and identified as such by Newton, Benedict & Dakin (1926) and by Eagles & Johnson (1927). It was somewhat later prepared from pig blood by Benedict, Newton & Behre (1926). Hunter (1928) described for the substance a delicate and highly specific colour test by which ergothioneine appeared to be present in significant quantity in the blood corpuscles of several animal species. Gulland & Peters (1930) recorded the isolation of ergothioneine from pigeon blood, and Williamson & Meldrum (1932) its isolation from pig blood. Eagles & Vars (1928) brought forward some evidence that the presence of ergothioneine in the blood of the pig was traceable to the diet, and more specifically to some constituent of maize. There has been no confirmation of the work of Eagles & Vars, partly on account of the fact that there was no satisfactory method for the determination of ergothioneine in blood.

The observations of Lawson & Rimington (1947) on an antithyroid activity of ergothioneine revived

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interest in the substance, and Hunter (1949) has described a new fractionation procedure to obtain blood filtrates suitable for ergothioneine determination by the diazo method.

The amounts of ergothioneine found in human, pig and rat blood are shown in Table 1, and a glance at the mean values shows a wide variation associated apparently with geographical location and particularly apparent in the values shown for pig blood.

The values given for Toronto, are calculated from those published by Hunter (1928). In that paper ergothioneine was expressed as mg./100 ml. corpuscles. In Table 1 of the present paper those values have been halved to give corresponding approximate values for whole blood. It can be seen that pig blood in the Toronto area in 1928 had more than three times the mean ergothioneine content of pig blood in the London area in 1949–50; and at present pig blood in the Edmonton area has about twice the concentration of pig blood in the London area. The relationship between Edmonton (Canada) and London (England) at present is similar with respect to human and rat blood.

There can be little doubt that such variations in ergothioneine blood level are real. They have been

Table 1. *Ergothioneine in blood (mg./100 ml. whole blood). Author's observations*

Date	Place	Species	No.	Mean	S.D.	Range
1949	Edmonton, Canada	Human	> 50	5.3	—	2.5– 9.5
1949	London, England	Human	20	2.9	0.75	1.8– 4.4
1928	Toronto, Canada	Pig	12	13.6	7.5	2.0–26.5
1949	Edmonton, Canada	Pig	> 10	7.8	—	6.0– 9.3
1949–50	London, England	Pig	13	4.4	0.74	3.3– 5.3
1949	Edmonton, Canada	Rat	> 10	6.1	—	2.9– 8.8
1950	London, England	Rat	15 (males)	1.9*	0.43	1.3– 2.6
1950	London, England	Rat	15 (females)	2.4*	0.53	1.3– 3.1

* This difference is significant; $t=3.12$ when $P<0.01$.

found by the same worker with the same method. The earlier method of Hunter (1928) made no claim to be precise especially for bloods in which the ergothioneine concentration was low, but with the pig bloods commonly handled in Toronto in 1927 the values published are probably substantially accurate, and at any rate represent minimum rather than maximum values. In support of this view it may be noted that Hunter & Eagles (1925) commonly isolated about 0.7 g. of pure ergothioneine from about 10 l. of pig blood, or a yield of 7 mg./100 ml. whole blood; and it is unlikely that such a yield represented more than 50 % of the substance actually present. Hunter, Molnar & Wight (1949) record that in the preparation of ergothioneine from pig blood in Edmonton their best yields were little better than 3 mg./100 ml., and several workers in Britain have failed to prepare ergothioneine from pig blood (personal information). It was also noted by Eagles & Vars (1928) that there appeared to be marked differences in the ergothioneine level of bloods of pigs from different regions of the United States of America.

In view of the wide differences in ergothioneine blood level thus established, and also because of its possible action on the thyroid gland, an attempt to find some explanation for the origin of ergothioneine in blood has here been made. There was strong circumstantial evidence that diets were responsible for the large differences found in the blood level of ergothioneine, and the 'maize theory' of Eagles & Vars (1928) had several points in its favour. Pigs fed maize had a high blood ergothioneine level, and it was suggested by Eagles & Vars (1928) that hydrolysed zein yielded a diazo-giving material that contained a thiolglyoxaline nucleus—possibly in the form of thiolhistidine—as suggested by a positive diazo test (Hunter, 1928, 1930).

Accordingly, it was decided to undertake feeding experiments with pigs and rats, and at the same time to investigate the diazo-giving material extractable from maize meal with methanol. As the present feeding experiments have failed to establish the existence of an ergothioneine precursor in maize, the

chemical investigation of maize here undertaken may not appear to be relevant to the main problem of explaining the differences in the level of ergothioneine of bloods. It is useful, however, to know that three distinct substances in maize give diazo tests. As the investigation had to be terminated at the present stage, it has not been possible to identify the substances, but the information obtained may be of some use to future workers in this field.

EXPERIMENTAL

Diazo test. (1) *Diazo reagent* (Koessler & Hanke, 1919). In a 50 ml. volumetric flask immersed in ice water is placed 1.5 ml. of a solution containing 9 g. sulphanilic acid and 90 ml. 37 % HCl/l., followed by 1.5 ml. 5 % NaNO₂. Allow to stand 5 min. then add further 6 ml. NaNO₂ solution. After further 5 min. make up to volume with ice-cold water and mix. The reagent must be kept cold.

(2) *Alkaline buffer.* 1 g. anhydrous Na₂CO₃ and 10 g. anhydrous sodium acetate in 100 ml. water.

(3) *10N-Sodium hydroxide.* If x ml. test solution are used put $2-x$ ml. water in tube in ice water followed by 1 ml. diazo reagent and 2 ml. alkaline buffer. Add x ml. test solution at once and mix. A clear lemon-yellow colour develops in the presence of ergothioneine. Allow 45 sec. for coupling then remove from ice water and run in 5 ml. 10N-NaOH and mix. Warm by holding in hand. In 3–4 min. the solution is free of air bubbles and the final purplish-pink colour has fully developed. This colour fades slowly.

Determination of ergothioneine in whole blood. Hunter (1949).

A. Feeding experiments

(a) *Pigs.* Through the kindness of Prof. H. D. Kay, Director, The National Institute for Research in Dairying, Reading, it has been possible to test the effect of a maize diet on the ergothioneine level of pig blood. Eight female pigs of average weight 45 lb., and from two litters, were divided into two groups of four, one of which was fed diet *M* and the other diet *O* of composition:

	Diet <i>M</i>	Diet <i>O</i>
Maize meal	77	—
Fine wheat offal	10	45
Barley meal	—	42
Fish meal	10	10
Dried yeast	3	3
Cod liver oil	30 g. once weekly	

Dr R. Braude was in charge of the experiment and sent oxalated blood samples, about 2 ml. from each of the pigs, on the dates specified in Table 2. On both diets the pigs grew at a fairly satisfactory rate, and on 17 April, at the end of 3 months on the diets, the average weight in both groups was 141–144 lb.

The results of the experiment are recorded in Table 2, ergothioneine being determined in the blood by the method of Hunter (1949) from a calibration curve determined for the Pulfrich potometer using filter no. 4.

Comment. For about 3 weeks before the start of the experiment all the pigs had been on a diet containing about 20% maize. It is clear that increasing the maize to 77% in the ration did not increase the ergothioneine in the blood, but did maintain the level better than diet *O*, containing no maize. Although the loss of ergothioneine from the blood on diet *O* is statistically significant, the level of ergothioneine, just over 4 mg./100 ml. whole blood for the pigs on diet *M*, quite obviously does not account for the discrepancies discussed in Table 1, and fails to confirm the suggestion of Eagles & Vars (1928) that dietary maize contains a blood-ergothioneine precursor.

(b) *Rats.* Thirty albino rats were taken, fifteen male and fifteen female, that had been raised on a maize-free diet composed of whole wheat 45, Sussex ground oats 40, fishmeal

8, dried skim milk 3, cod liver oil 2, dried yeast 1, and salt 1. Six of the animals, three male and three female were anaesthetized, bloods removed by puncture of the exposed hearts, and the animals killed. The blood ergothioneine values of these control animals are recorded in Table 3.

The remaining twenty-four rats were divided into four cages of six male and six female and fed diets *A* and *B* of composition:

	Diet A	Diet B
Maize meal	80	—
Methanol-washed maize meal	—	76
Casein	10	10
Olive oil	—	4
Glaxo salt mixture	4	4
Dried yeast	5	5
Cod liver oil	1	1

Maize meal in lots of about 1.5 kg. was washed in a percolator at room temperature with methanol until the extract was almost colourless and no longer gave a positive diazo test. The dried extracted material, free from methanol, is the constituent referred to as methanol-washed maize in rat diet *B*.

On the dates specified in Table 3 one rat was removed from each cage, the blood taken and the animal killed as described for the controls. The ergothioneine values are recorded in Table 3.

Comment. The mean ergothioneine values of the blood of rats fed maize over a period of 15 weeks are

Table 2. *Ergothioneine in blood of pigs fed maize (diet M) and no maize (diet O)*
(mg. ergothioneine/100 ml. whole blood)

Ear mark	17 Jan.	30 Jan.	9 Feb.	20 Feb.	27 Mar.	17 Apr.
			Diet <i>M</i>			
10†	5.3	5.3	5.3	5.7	5.7	4.8
11†	5.3	4.8	4.8	4.8	4.4	4.0
22‡	4.2	4.0	4.4	4.4	4.4	3.5
14‡	5.3	5.3	5.3	5.3	4.8	4.4
	Mean 5.0*					Mean 4.2*
			Diet <i>O</i>			
08†	5.3	4.8	4.0	4.0	3.5	3.3
09†	4.8	4.0	3.5	3.5	2.9	3.5
21‡	4.4	4.0	3.5	3.5	3.3	3.1
23‡	Clotted	4.4	3.5	3.1	3.3	2.9
	Mean 4.8**					Mean 3.2**

* Difference not significant; $t=1.95$, $P>0.05$.

** Difference significant; $t=6.12$, $P<0.01$.

† Litter mates.

‡ Litter mates.

Table 3. *Ergothioneine in blood of rats fed no maize (control), maize (diet A), and methanol-washed maize (diet B) (mg. ergothioneine/100 ml. whole blood)*

Diet	Sex	16 Jan.	26 Jan.	6 Feb.	16 Feb.	15 Mar.	24 Apr.	2 May	
Control	M	1.6, 1.5, 1.3	Mean (6 rats) = 1.6*						
	F	2.2, 1.7, 1.3							
A	M	—	2.4	1.5	2.0	2.4	1.5	1.8	Mean (12 rats) = 2.3*
A	F	—	3.1	2.9	2.6	3.1	2.0	2.2	
B	M	—	2.6	1.8	2.0	2.6	2.0	1.5	Mean (12 rats) = 2.3*
B	F	—	2.9	2.6	2.6	2.9	2.1	2.0	

* This difference is significant; $t=3.3$ or $P<0.01$.

N.B. The last eight values for 24 April and 2 May have mean = 1.9. This is not significantly different from controls; $t=1.84$ or $P>0.05$.

significantly higher than corresponding values on rats fed a maize-free diet, although the mean value of the last eight rats killed was not significantly higher than the controls. There is no evidence that washing the maize meal with methanol removes any ergothioneine precursor. As in the pig experiments, the findings fail to account for the discrepancies noted in Table 1.

B. Diazo-reacting substances in maize

It has been observed that, besides ergothioneine, other substances containing the thioglyoxaline nucleus give a clear yellow colour on coupling with the alkaline diazo reagent, and on the addition of strong base the yellow colour is changed to orange or red (Hunter, 1930). As has been noted above, Eagles and Vars, on the basis of this test, suggested that there was a thioglyoxaline present in zein. Ashley & Harington (1930) note that they searched for 2-thiohistidine in zein and I have examined zein, hydrolysed with 20% hydrochloric acid, without finding evidence of the presence of a thioglyoxaline nucleus.

When a little maize meal powder is mixed with the alkaline diazo reagent used to test for ergothioneine a pinkish colour appears which acquires a more intense red on the addition of concentrated sodium hydroxide solution. This diazo-giving material of maize is extractable with methanol and thus probably not protein in character. Here appeared a possible explanation of the claim of Eagles & Vars that maize contained a dietary precursor of the ergothioneine of blood, which precursor had not been proved to be present in zein. The nature of the diazo-giving material in maize was thought worth investigating while the nutritional tests on pigs and rats, described above, were progressing. It was also for this reason that one group of rats was fed maize that had been freed from the diazo-giving substance by washing with methanol.

Fractionation of methanol extract. The methanol extract from about 1.5 kg. maize meal, vol. 4-5 l., was treated as follows:

The bulk of the methanol was distilled off at atmospheric pressure on a boiling-water bath to reduce the volume to about 500 ml. Distillation was continued *in vacuo* to reduce the aqueous fatty emulsion to about 100 ml. As the maize meal was not dried to begin with there was appreciable water in the extract, but after vacuum distillation it was quite free from methanol. Light petroleum, b.p. 40-60°, was added copiously to the flask and the mixture transferred to a separating funnel. The brown light petroleum layer, containing much fat and giving no diazo test, was discarded. Washing was repeated to remove all light petroleum-soluble material. There was left a light brown solid suspended in an aqueous phase. The latter was separated and collected as a 'water-soluble fraction' which gave a marked diazo test (see below). The particulate material was washed several times

with water, and then dissolved in a minimum volume of methanol. A drop of this methanol solution gave a strong red coupling colour with the diazo reagent, and on the addition of conc. NaOH the colour became more intense and of a blue tinge. To the methanol solution about an equal volume of ethanol was added. This produced a copious precipitate with a tendency to gum formation. The methanol-ethanol mixture, which contained all of the diazo-giving material, was evaporated to dryness *in vacuo* and the residue was extracted with absolute ethanol. The ethanol extract was evaporated to dryness and the dry residue extracted with ethyl ether which removed a yellow pigment but no diazo-giving material. The dry light brown friable material was dissolved in about 25 ml. absolute ethanol and hot aqueous Ba(OH)₂ was added until there was no further precipitation. The mixture was centrifuged and the material well washed with absolute ethanol.

The precipitate, which contained the substance sought, was now extracted with water which dissolved its Ba salt. CO₂ was passed through the clear yellow solution and the mixture centrifuged and washed with CO₂-saturated water. The white precipitate was now well drained from water and extracted with ethanol. This dissolved the free water-insoluble substance sought and left insoluble BaCO₃ and certain Ba salts not freed with CO₂. The ethanol-soluble material was evaporated to dryness *in vacuo* to give an anhydrous residue. Yield about 200 mg. This is referred to as substance 1.

Three separate preparations are described below.

Preparation C was obtained by the above method, and preparation B by repeating the fractionation with Ba(OH)₂, ethanol, water and CO₂. Preparation A was obtained by further treatment. The material, about 150 mg. dissolved in about 5 ml. ethanol, was absorbed on a column of Al₂O₃ and well washed with ethanol. No appreciable material was separated in this way. The column was then extracted with 0.5N-NaOH in ethanol. The clear yellow eluate, giving an intense diazo test, was made just acid with conc. HCl and the NaCl removed. The filtrate, as clear and colourless as water, was brought to dryness *in vacuo*, and the residue extracted several times with water to remove all NaCl. The nearly white precipitate was then dissolved in ethanol and the solution brought to dryness *in vacuo*. The material was again taken up in ethanol and dried to an anhydrous light brown residue that was readily removed from the tube.

After elution with 0.5N-NaOH in ethanol, considerable brown material was left on the column, so that preparation A is purer than B or C. A small amount of the material was paper chromatographed with a collidine-lutidine mixture. When sprayed with Na₂CO₃ solution a yellow spot was perceptible before application of the diazo reagent, indicating that the substance is intrinsically yellow in alkaline solution. It gave an intense red on application of the diazo reagent. (*R_F* about 0.95.) Correct conditions for crystallization of the substance have not been determined. It was observed that 11.4 mg. of the substance completely dissolved in 3 ml. 0.02N-NaOH, but on standing overnight the contents of the test tube became cloudy due to some amorphous material, but also to numerous glistening rectangular plate-like crystals. The pH of such a solution would be near the pK of the substance (see below). All attempts at crystallization by the usual methods have yielded emphatically amorphous precipitates.

Properties of substance 1

M.p.'s: preparation *A*, 120°; *B*, 118–121°; *C*, 120–122° (uncorrected). The substance burns without ash and tests for halide, S and P were negative. (The substance was fused with Na for the S test.) Analyses of the three preparations are as follows:

Preparation *A*: C, 61.9; H, 6.5; N, 6.0%

Preparation *B*: C, 63.1; H, 6.6; N, 5.4%

Preparation *C*: C, 63.3; H, 6.6; N, 5.8%

$C_{13}H_{17}O_4N$ required C, 62.1; H, 6.8; N, 5.6%.

The substance (59.1 mg.) was dissolved in 17 ml. 0.02N-NaOH, the solution having a pH of 11.3. When titrated with 0.02N-HCl precipitation began at pH 9.7 and was complete at pH 8.0. The pK is about 9.1.

The substance (45 mg.) was refluxed with 10 ml. 20% HCl for 10 hr. The material never went into solution, but formed black carbonized flecks and all the diazo-giving substance was destroyed.

Solubilities. The substance is soluble in methanol, ethanol and butanol. It is insoluble in water and in dilute and strong acids. It is insoluble in $NaHCO_3$ and Na_2HPO_4 , but soluble in Na_2CO_3 , Na_3PO_4 and NaOH solutions. It is insoluble in ether, chloroform and light petroleum.

Tests. Molisch, Fehling, biuret and ninhydrin tests were negative. The substance gives a positive xanthoproteic test, a blue colour with uric acid reagents, and a deep-green colour in ethanol with $FeCl_3$. It gives an incipient Millon's test and a burgundy-coloured solution when heated with 50% H_2SO_4 .

A few mg. of the substance added to the alkaline Koessler & Hanke (1919) diazo reagent gives an immediate red colour which quickly fades to yellow. However, if 10N-NaOH solution is quickly added the red colour acquires a bluish tinge and persists for some hours.

With the buffered thiolglyoxaline diazo reagents the behaviour is similar.

Bromine water is rapidly decolorized by the substance. When a slight excess of bromine has been added the solution no longer gives a diazo test.

Dilute $KMnO_4$ solution is rapidly decolorized in presence of H_2SO_4 by the substance. When excess $KMnO_4$ has been added the solution shows a negative diazo test.

Solutions of the substance in NaOH or Na_2CO_3 absorb strongly in the ultraviolet region. Preparations *A*, *B* and *C* were examined in a concentration of 0.8 mg./100 ml. in 2% (w/v) Na_2CO_3 solution. In all cases the maximum optical densities were at 355 $m\mu$, and in a 1 cm. depth, of magnitudes 0.68, 0.63 and 0.61 respectively. After about 12 hr. the peak optical densities were found to have dropped to 75–80% of the values found in the freshly prepared solutions. Whether this drop is due to tautomerism or decomposition was not determined.

When the substance is examined with just enough base to hold it in aqueous solution the absorption maximum is near 290 $m\mu$.

The above evidence, particularly the analyses and absorption spectra of the three separate preparations, would suggest that the substance has been obtained in a state approaching purity. It would appear that the substance is phenolic in character although the possibility of the presence of a carboxyl group is not

excluded. The absence of sulphur definitely excludes the presence of a thiolglyoxaline, and a glyoxaline nucleus is also almost certainly excluded on the evidence of the low percentage of nitrogen and on its behaviour towards the diazo reagents.

The behaviour of the substance towards the diazo reagents is atypical with respect to the intensification of colour produced by the addition of strong alkali. This is not usually produced either with phenols or glyoxalines, though a similar behaviour is found with α - and β -naphthol and with resorcinol. The very rapid development of colour with the Koessler & Hanke (1919) reagents is typical of phenols. It should thus be noted that intensification of colour on the addition of a strong base is not in itself indicative of a thiolglyoxaline test; a yellow coupling colour is also required.

The substance would appear to be unusual in composition and properties, and further study is necessary to determine its constitution, but the present evidence suggests that it is not a precursor of ergothioneine.

Water-soluble fraction. The water-soluble fraction obtained as described above gives an immediate brownish-red colour with the diazo reagent which is intensified to a clear red on the addition of 10N-NaOH. When the water-soluble fraction, suitably diluted and at pH about 4, is treated with 20% (w/v) lead acetate a small yellowish precipitate containing little diazo-giving material is obtained. When the filtrate from this is brought to pH 9.0 by addition of NaOH a copious precipitate is obtained. When this is freed from Pb by H_2SO_4 the solution gives a dull red diazo test, intensified to a bright red with NaOH. The substance giving this test will here be referred to as substance 2. The filtrate from the Pb precipitate, when freed from Pb by H_2SO_4 , gives a yellow diazo test with little intensification by NaOH. This will be referred to as substance 3.

Both substances 2 and 3 are precipitable by phosphotungstic acid. When their phosphotungstates are freed quantitatively with $Ba(OH)_2$ and (H_2SO_4) the solution containing substance 2 is neutral and that containing substance 3 is strongly basic.

Some properties of substances 2 and 3, inferred from examination of such solutions, are shown in Table 4.

It would thus appear that both substances are basic in character and that substance 2 is phenolic. The possibility remains that substance 3 is a thiolglyoxaline. Its diazo test is slight evidence for such a possibility, as certain thiolglyoxalines show only slight intensification of the yellow coupling colour on addition of strong base.

DISCUSSION

The uniformity of ergothioneine level in the blood of the eight female pigs and of the six control rats used is impressive. The slight downward trend in blood levels of the pigs is also marked by a regularity commonly associated with good analytical methods. In this connexion, however, it should again be emphasized that the low values here recorded are

Table 4. *Some properties of substances 2 and 3*

Substance 2	Substance 3
Neutral	Basic
Precipitated with phosphotungstic acid	Precipitated
Precipitated with lead acetate at pH 9.0	Not precipitated
Diazo test, dull-red intensified by NaOH	Yellow, little change with NaOH
Uric acid reagent, blue	No colour
Molisch test, strongly positive	Strongly positive
Decolorizes KMnO_4	Decolorizes KMnO_4
Boiled with 5N-HCl, colourless	Rapid blackening with 'humin' formation
Addition of I_2 , brown precipitate	Dark-brown precipitate
Dragendorff's reagent, yellow precipitate	Dark-brown precipitate
Spectral absorption band at max. 290 m μ .	No band

higher, by about 1.0 mg., than the real ergothioneine content of the bloods examined as no adjustment has been made for plasma blanks (Hunter, 1949). A significant yellow colour with the diazo reagent is given by plasma, probably due to histidine and tyrosine. On the other hand, it may be stated that no blood has been examined without evidence by the diazo test of the existence in it of some ergothioneine.

In the case of the twenty-four experimental rats it is assumed that the blood level was the same as the six controls. This is a fairly risky assumption as a slight increase in the control values would render insignificant the statistical significance noted in Table 2. But assuming the reality of a statistical significance here, it is relatively uninteresting in the light of the known much greater physiological differences observed in different geographical regions.

The significant sex difference in rats noted in Table 1, although not large, is probably real, and under more favourable conditions might be increased.

It may be noted that the observations on the chemistry of diazo-reacting substances in maize are in accord with the findings in the feeding experiments in so far as they provide no evidence for the existence of a thioglyoxaline nucleus. The possibility remains that American-grown maize differs in composition from that grown in other regions. The source of the maize used in the present experiments could not be traced.

The problem of why bloods should differ so

markedly in ergothioneine level thus remains quite obscure. But the subject has scarcely been investigated. A preliminary observation would indicate that when ergothioneine or thiolhistidine is fed to rats, 5 mg./rat for 3 days, the blood ergothioneine 2 days later is not increased.

Pirie (1933) has shown that ergothioneine catalyses the oxidation of cysteine by hydrogen peroxide at pH 2.0, like certain other substances containing the SH-group.

SUMMARY

1. The level of ergothioneine in the blood of the human, pig and rat is much lower in London, England, than in Edmonton, Canada.

2. The thesis of Eagles & Vars (1928) that maize in the diet contains a precursor of ergothioneine of blood has not been confirmed.

3. It has been shown that there are three substances extractable from maize by methanol that give positive diazo tests, none of which is typical of the presence of thioglyoxaline.

I would express to Prof. C. Rimington my deepest appreciation for inviting me to be a guest in his Department, and my thanks to its staff for assistance in many ways.

The spectral absorption data recorded were determined with the aid of a Beckman photoelectric spectrophotometer purchased out of a grant made to Prof. Rimington from the University of London Central Research Grant Fund.

Acknowledgement is made to the Medical Research Council for a personal grant for this research.

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