Oxidative DNA damage induced by homogentisic acid, a tyrosine metabolite

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Abstract We examined the mechanism of DNA damage induced by a mutagenic tyrosine metabolite, homogentisic acid (HGA), using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene. HGA caused DNA damage in the presence of Cu(II), particularly at thymine and cytosine residues. Catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine by HGA increased depending on HGA concentration in the presence of Cu(II). It is concluded that H_2O_2 is generated during Cu(II)-catalyzed HGA autoxidation and reacts with Cu(I) to form the Cu(I)-peroxide complex, capable of causing oxidative DNA damage.

Key words: Homogentisic acid; Tyrosine metabolite; DNA damage; Active oxygen species; Hydrogen peroxide; Copper

1. Introduction

Dietary factors account for approximately 35% of cancers attributed to environmental factors [1,2]. One possible example of dietary carcinogenesis is the involvement of proteins, amino acids and their metabolites. Epidemiological studies have suggested that high protein intake is a risk factor for several types of cancers, particularly colon, rectum and breast cancers [3,4]. Significantly increased risks of renal cell cancer were observed with increasing consumption of high protein foods [5]. An animal experiment has demonstrated that high protein intake enhanced the development of chemically induced preneoplastic foci [6]. However, the mechanism of carcinogenesis caused by protein intake remains to be clarified.

The possibility that tyrosine metabolites may participate in carcinogenesis has been pointed out. It has been suggested that tyrosine is a rate-limiting substrate for tumor cell proliferation [7]. Tyrosine is metabolized to homogentisic acid (HGA) through the formation of *p*-hydroxyphenylpyruvate and *p*-hydroxyphenyllactic acid. HGA has been reported to be mutagenic in bacteria and mammalian cells [8]. *p*-Hydroxyphenyllactic acid induced leukemic changes and hepatomas in mice [9,10], suggesting that HGA may be involved in causing malignant tumors. A high incidence of hepatocarcinoma was observed in patients with the chronic form of hereditary tyrosinemia type I [9,11]. When HGA was administered orally, the plasma level of HGA in carriers of hereditary tyrosinemia type I was significantly higher than that in controls [12]. Therefore, accumulation of HGA in patients with this disease may play an important role in causing malignant tumors.

To investigate the involvement of HGA in carcinogenesis, we examined DNA damage induced by HGA using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene. It has been suggested that some dietary factors can exert prooxidant actions [13,14] and may be involved in oxidative DNA damage. Therefore, we measured the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative damage to DNA, using an electrochemical detector coupled to HPLC (HPLC-ECD).

2. Materials and methods

2.1. Materials

Restriction enzyme *Hin*dIII was purchased from New England Biolabs. Restriction enzymes (*Eco*RI and *Apa*I) and calf intestine phosphatase were from Boehringer Mannheim GmbH. [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. HGA was from Nacalai Tesque Co. (Kyoto, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproine disulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA, superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan).

2.2. Preparation of ^{32}P-5′-end-labeled DNA fragments

DNA fragments were obtained from the human *p53* tumor suppressor gene [15]. The ^{32}P-5′-end-labeled 650-bp DNA fragment (*Hin*dIII* 13 972–*Eco*RI* 14 621) was obtained as described previously [16]. The 650-bp fragment was digested with *Apa*I to obtain a singly labeled 443-bp (*Apa*I 14 179–*Eco*RI* 14 621) and 211-bp (*Hin*dIII* 13 972–*Apa*I 14 182) DNA fragments. The asterisk indicates ^{32}P labeling.

2.3. Detection of damage to ^{32}P-labeled DNA fragments

The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained HGA, [^{32}P]DNA fragment and 10 μM/base sonicated calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation for 30 min at 37°C, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously [17,18]. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure [19] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

2.4. Measurement of 8-oxodG formation by HGA

The amount of 8-oxodG was measured by a modified method of Kasai et al. [20]. The reaction mixtures containing 100 μM/base calf thymus DNA fragments, HGA and 20 μM $CuCl_2$ in 4 mM sodium phosphate buffer (pH 7.8) containing 2 μM DTPA were incubated for 30 min at 37°C. After ethanol precipitation, DNA was digested to the

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Abbreviations: HGA, homogentisic acid; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; HPLC, high performance liquid chromatography; HPLC-ECD, electrochemical detector coupled to HPLC; DTPA, diethylenetriamine *N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase

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nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed with an HPLC-ECD as described previously [21].

2.5. UV-visible spectra measurement

UV-visible spectra were measured at 37°C with a UV-vis-NIR recording spectrophotometer (Shimadzu UV-365). The reaction mixture contained 100 μM HGA and 20 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Spectral tracing was initiated by addition of $CuCl_2$.

3. Results

3.1. Effects of scavengers and bathocuproine on DNA damage by HGA plus Cu(II)

Fig. 1 shows the autoradiogram showing the effects of hydroxyl free radical (•OH) scavengers, catalase, SOD and bathocuproine, a Cu(I)-specific chelator, on DNA damage induced by HGA plus Cu(II). HGA caused DNA damage in the presence of Cu(II) (lane 2), although DNA damage was not caused by HGA alone or Cu(II) alone (data not shown). HGA caused little or no DNA damage in the presence of Fe(II), Fe(III) or Mn(II) (data not shown). DNA damage was completely inhibited by catalase (lane 7) and 50 μM bathocuproine (lane 10), suggesting that hydrogen peroxide (H_2O_2) and Cu(I) were involved. Typical •OH scavengers, ethanol, mannitol and sodium formate, did not inhibit DNA damage (lanes 3–5), whereas methional showed an inhibitory effect (lane 6). SOD showed little or no inhibitory effect on DNA damage (lane 8). The intensity of DNA damage increased depending on HGA concentration and incubation time (data not shown). DNA damage was enhanced by piperidine treatment (data not shown), suggesting that HGA plus Cu(II) caused not only strand breakage but also base damage and liberation.

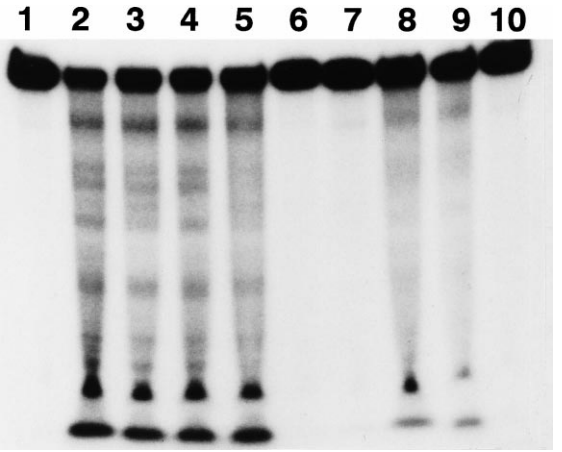

Fig. 1. Effects of scavengers and bathocuproine on DNA damage by HGA plus Cu(II). The reaction mixtures containing ^{32}P-5′-end-labeled 211-bp DNA fragments, 10 μM/base of sonicated calf thymus DNA, 10 μM HGA and 20 μM $CuCl_2$ in 200 μl of 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 30 min at 37°C. The DNA fragments were treated with 1 M piperidine for 20 min at 90°C and electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. Control (lane 1) contained neither HGA nor $CuCl_2$. Scavenger or bathocuproine was added as follows: lane 2, no scavenger; lane 3, 5% ethanol; lane 4, 0.1 M mannitol; lane 5, 0.1 M sodium formate; lane 6, 0.1 M methional; lane 7, 150 units/ml catalase; lane 8, 150 units/ml SOD; lane 9, 20 μM bathocuproine; lane 10, 50 μM bathocuproine.

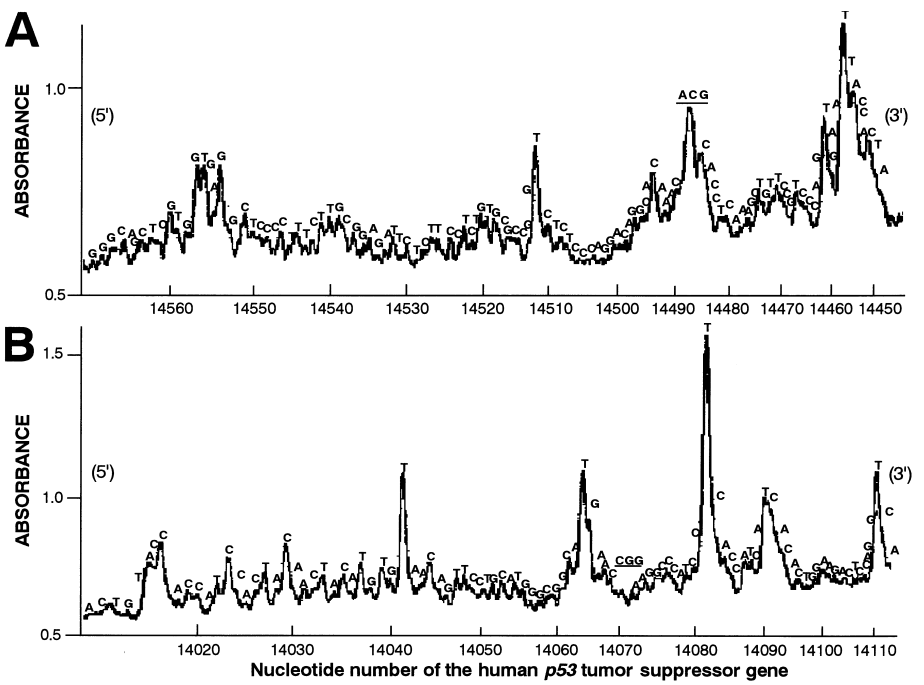

Fig. 2. Site specificity of DNA cleavage induced by HGA in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end labeled 443-bp (*Apa*I 14179–*Eco*RI* 14621) (A) or 211-bp DNA fragment (*Hin*dIII* 13972–*Apa*I 14182) (B), 10 μM/base of sonicated calf thymus DNA, 10 μM HGA and 20 μM $CuCl_2$ in 200 μl of 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation for 30 min at 37°C, the DNA fragments were treated by the method described in the legend to Fig. 1. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene. Underscoring, mutational hot spots in the *p53* gene.

3.2. Site specificity of DNA cleavage induced by HGA plus Cu(II)

To examine the DNA cleavage sites, ^{32}P-5′-end-labeled DNA fragments treated with HGA plus Cu(II) and subsequently with piperidine were electrophoresed and an autoradiogram was obtained as shown in Fig. 2. HGA induced piperidine-labile sites particularly at thymine and cytosine residues, although there remains a possibility that certain base damage might be over- or underrepresented dependent on its sensitivity to piperidine.

3.3. Formation of 8-oxodG induced by HGA

Using HPLC-ECD, we measured the contents of 8-oxodG in calf thymus DNA treated with HGA, and the result is shown in Fig. 3. The formation of 8-oxodG increased depending on HGA concentration in the presence of Cu(II). In the absence of Cu(II), the content of 8-oxodG did not increase.

3.4. Changes in the UV-visible spectrum of HGA

In the presence of Cu(II), a rapid change in the spectrum of HGA was observed and the maximum absorption at 248 nm increased with time. Little or no spectral change was observed in the absence of Cu(II) or in the presence of Fe(II) or Fe(III) (data not shown).

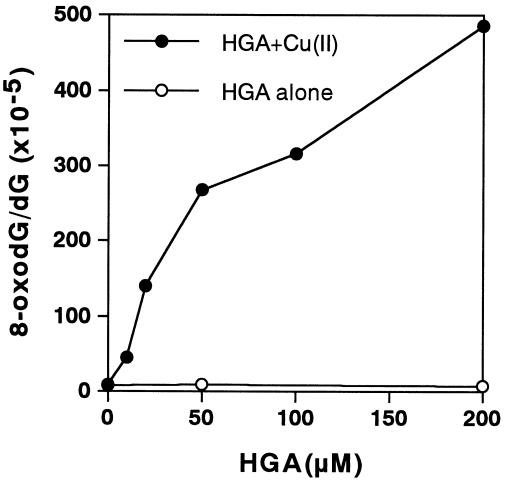

Fig. 3. Formation of 8-oxodG induced by HGA plus Cu(II). The reaction mixture contained 100 μM/base calf thymus DNA, HGA and 20 μM $CuCl_2$ in 400 μl of 4 mM phosphate buffer (pH 7.8) containing 2 μM DTPA. After incubation for 30 min at 37°C, 0.2 mM DTPA was added to stop the reaction and the DNA was precipitated in ethanol. The DNA fragment was enzymatically digested into nucleosides, and 8-oxodG formation was measured BY HPLC-ECD.

4. Discussion

In the present study, we have demonstrated that a low concentration of HGA induced damage to DNA fragments obtained from the *p53* tumor suppressor gene. Inhibitory effects of catalase and bathocuproine on DNA damage by HGA plus Cu(II) suggest the involvement of H_2O_2 and Cu(I). Typical $^{\bullet}OH$ scavengers showed little or no inhibitory effect on DNA damage suggesting that $^{\bullet}OH$ or, at least, free $^{\bullet}OH$ does not play an important role in DNA damage. The DNA damage was inhibited by methional, which scavenges a variety of free radicals including active species resembling $^{\bullet}OH$ [22,23]. HGA plus Cu(II) caused DNA damage at thymine and cytosine residues. The site specificity supports the involvement of active oxygen species other than $^{\bullet}OH$, because $^{\bullet}OH$ causes DNA cleavage at any nucleotides with little site specificity [24,25].

The proposed mechanism of DNA damage induced by HGA plus Cu(II) is shown in Fig. 4. HGA undergoes Cu(II)-mediated oxidation to the semiquinone radical, which would be further oxidized to benzoquinone acetate. It has been reported that HGA undergoes oxygen-dependent autoxidation to generate active oxygen species [26]. During the autoxidation of HGA, Cu(II) is reduced to Cu(I). Superoxide anion (O_2^-) is generated by the reaction of O_2 with Cu(I) or the semiquinone radical, and then dismutated to H_2O_2. H_2O_2 reacts with Cu(I) to form a crypto-OH radical, such as the Cu(I)-peroxide complex, capable of causing site-specific DNA damage. However, there remains the possibility that the site specificity of DNA damage can be conferred by binding of copper to DNA at specific sites.

HGA plus Cu(II) caused DNA damage at the cytosine residue in the 5′-A<u>C</u>G-3′ sequence, which is complementary to the sequence of codon 273 (5′-CGT-3′). Codon 273 is known to be a mutational hot spot of the *p53* gene [27,28]. Oxidative damage to DNA would participate in mutations of the *p53*

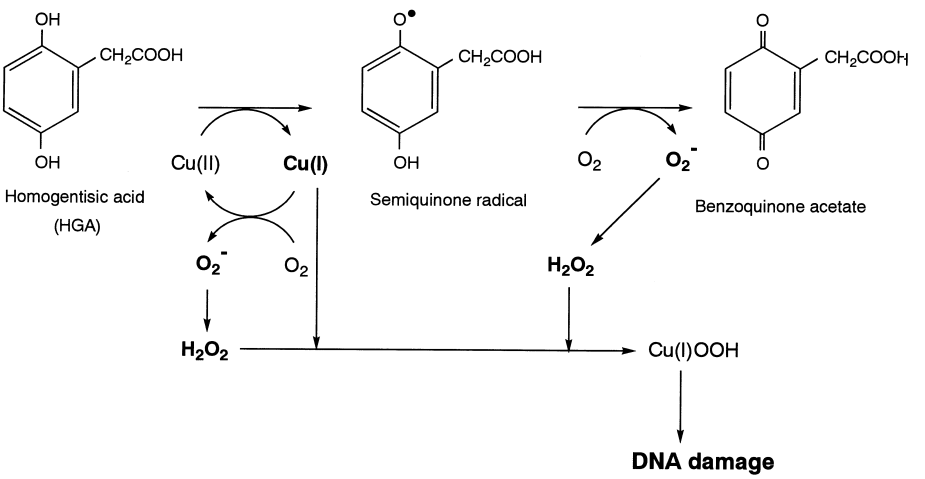

Fig. 4. Proposed mechanism of DNA damage induced by HGA plus Cu(II).

gene, leading to carcinogenesis. The present study demonstrated that HGA caused oxidative DNA damage, including the formation of 8-oxodG, an oxidative product of DNA, in the presence of Cu(II). Shibutani et al. have reported that formation of 8-oxodG causes DNA misreplication which can lead to mutation, particularly G→T substitution [29].

Copper occurs in the mammalian cell nucleus, and may contribute to high-order chromatin structures [30]. Copper ion binds to non-histone proteins, and causes much stronger ascorbate-mediated DNA damage than iron [31]. Copper has the ability to catalyze the production of active oxygen species and to mediate oxidative DNA damage [32–34]. These studies support the observation that HGA caused DNA damage through generation of active oxygen species by the interaction with copper.

Alterations in amino acid metabolism can be caused by high protein intake, amino acid-imbalanced diet and metabolic disorders. A causal relationship between alterations in amino acid metabolism and carcinogenesis has been suggested. Disturbance of tyrosine metabolism is associated with carcinogenesis in patients with tyrosinemia [9,11]. Tyrosine metabolites, including HGA and its precursors, have shown mutagenicity [8] and carcinogenicity [10]. This study has demonstrated that HGA caused DNA damage by generating active oxygen species. We have previously reported that certain tryptophan metabolites induced oxidative DNA damage [35]. Abnormal tryptophan metabolism is associated with bladder cancer [36,37]. There may be a number of amino acid metabolites capable of causing oxidative DNA damage, as well as HGA and tryptophan metabolites. Further investigation of the carcinogenic effects of amino acid metabolites is needed to clarify the mechanism of carcinogenesis induced by dietary protein intake and disturbance of amino acid metabolism.

References

[1] Doll, R. and Peto, R. (1981) J. Natl. Cancer Inst. 66, 1191–1308.
[2] Pitot, H.C. and Dragan, Y.P. (1996) in: Chemical Carcinogenesis (Klaassen, C.D., Ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons, pp. 201–267, McGraw-Hill, New York.
[3] Armstrong, B. and Doll, R. (1975) Int. J. Cancer 15, 617–631.
[4] Hems, G. (1978) Br. J. Cancer 37, 974–982.
[5] Chow, W.H., Gridley, G. and McLaughlin, J.K. (1994) J. Natl. Cancer Inst. 86, 1131–1139.
[6] Youngman, L. and Campbell, T. (1992) Nutr. Cancer 18, 131–142.
[7] Bartlett, D.L., Ste, T.P. and Torosian, M.H. (1995) Surgery 117, 260–267.
[8] Glatt, H. (1990) Mutat. Res. 238, 235–243.
[9] Laberge, C., Lescault, A. and Tanguay, R.M. (1986) Adv. Exp. Med. Biol. 206, 209–221.
[10] Rauschenbach, M.O., Zharova, E.I., Sergeeva, T.I., Ivanova, V.D. and Probatova, N.A. (1975) Cancer Res. 35, 577–585.
[11] Weinberg, A.G., Mize, C.E. and Worthen, H.G. (1976) J. Pediatr. 88, 434–438.
[12] Laberge, C., Lescault, A., Grenier, A., Morrisette, J., Gagne, R., Gadbois, P. and Halket, J. (1990) Am. J. Hum. Genet. 47, 329–337.
[13] Halliwell, B. (1996) Free Radical Res. 25, 57–74.
[14] Halliwell, B. (1996) Annu. Rev. Nutr. 16, 33–50.
[15] Chumakov, P. (1990) EMBL Data Library, accession number X54156.
[16] Yamashita, N., Murata, M., Inoue, S., Hiraku, Y., Yoshinaga, T. and Kawanishi, S. (1998) Mutat. Res. 397, 191–201.
[17] Yamamoto, K. and Kawanishi, S. (1991) J. Biol. Chem. 266, 1509–1515.
[18] Kawanishi, S. and Yamamoto, K. (1991) Biochemistry 30, 3069–3075.
[19] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[20] Kasai, H., Crain, P.F., Kuchino, Y., Nishimura, S., Ootsuyama, A. and Tanooka, H. (1986) Carcinogenesis 7, 1849–1851.
[21] Ito, K., Inoue, S., Yamamoto, K. and Kawanishi, S. (1993) J. Biol. Chem. 268, 13221–13227.
[22] Youngman, R.J. and Elstner, E.F. (1981) FEBS Lett. 129, 265–268.
[23] Pryor, W.A. and Tang, R.H. (1978) Biochem. Biophys. Res. Commun. 81, 498–503.
[24] Kawanishi, S., Inoue, S. and Sano, S. (1986) J. Biol. Chem. 261, 5952–5958.
[25] Celander, D.W. and Cech, T.R. (1990) Biochemistry 29, 1355–1361.
[26] Martin Jr., J.P. and Batkoff, B. (1987) Free Radical Biol. Med. 3, 241–250.
[27] Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) Science 253, 49–53.
[28] Denissenko, M.F., Pao, A., Tang, M. and Pfeifer, G.P. (1996) Science 274, 430–432.
[29] Shibutani, S., Takeshita, M. and Grollman, A.P. (1991) Nature 349, 431–434.
[30] Burkitt, M.J. (1994) Methods Enzymol. 234, 66–79.
[31] Chiu, S., Xue, L., Friedman, L.R. and Oleinick, N.L. (1995) Biochemistry 34, 2653–2661.
[32] Kawanishi, S., Ito, K., Oikawa, S., Yamamoto, K. and Inoue, S. (1994) in: Frontiers of Reactive Oxygen Species in Biology and Medicine (Asada, K. and Yoshikawa, T., Eds.), pp. 153–156, Excerpta Medica, Amsterdam.
[33] Guyton, K.Z. and Kensler, T.W. (1993) Br. Med. Bull. 49, 523–544.
[34] Li, Y. and Trush, M.A. (1994) Cancer Res. 54, 1895s–1898s.
[35] Hiraku, Y. and Kawanishi, S. (1995) Carcinogenesis 16, 349–356.
[36] Abul-Fadl, M.A.M. and Khalafallah, A.S. (1961) Br. J. Cancer 15, 478–482.
[37] Bryan, G.T. (1971) Am. J. Clin. Nutr. 24, 841–847.