Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine

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The formulation of the culture medium for a Chinese hamster ovary (CHO) cell line has been investigated in terms of the simultaneous replacement of glucose and glutamine, the most commonly employed carbon and nitrogen sources, pursuing the objective of achieving a more efficient use of these compounds, simultaneously avoiding the accumulation of lactate and ammonium in the medium. The key factor in this process is the selection of compounds that are slowly metabolized. Among the different compounds studied, galactose and glutamate provide the best results, allowing support of cell growth with an optimal balance between nutrient uptake and cell requirements and the generation of minimal quantities of lactate and ammonium. The attained results also highlight the capacity of the cells to reallocate their metabolism as a response to the changes in medium composition.

Introduction

The formulation of optimal culture media for animal cell growth has received a lot of interest for many years as a result of its critical impact on the development of bioprocesses based on animal cell technology (Sato, 1975; Kovar and Franck, 1986; Castro et al., 1992; Keen and Rapson, 1995; Lao and Shalla, 1996). Undoubtedly, the replacement of fetal calf serum is the most noteworthy advancement, particularly if it can be achieved by media containing low protein concentration or media that are completely protein-free. When this is achieved, the process can be operated with a medium that is well defined, that is, with constant quality and composition, and this has a major impact in terms of process reproducibility, downstream processing, and sometimes in the medium cost. Also, from the point of view of regulation and acceptability of the final product, especially if it has a therapeutical use, it will be an increasingly important requisite to grow the cells in media without any components originated from an animal source, such as fetal calf serum. All of these reasons have generated a lot of interest, and various successful formulations have been developed, leading to a number of protein-free and serum-free media that are commercially available today. In addition to these efforts, the reformulation of media with different types of compounds, such as vitamins, growth factors, hormones, lipids, trace elements, or amino acids, has been addressed. An important criterion in the development of new media is, in addition to these efforts, the reformulation of media with different types of compounds, such as vitamins, growth factors, hormones, lipids, trace elements, or amino acids, which has been addressed. An important criterion in the development of new media is, in addition to the supplementation of all the necessary nutrients, to do it in a balanced way, avoiding unnecessary increase in the osmolarity of the medium and also in the generation of metabolic waste products (Castro et al., 1992; Xie and Wang, 1994; Lao and Shalla, 1996).

In this effort for medium reformulation, it has been generally accepted that the compounds to use in the medium as main carbon and energy sources are glucose and glutamine. However, the rapid glucose and glutamine metabolism that animal cells exhibit when they are cultured in vitro leads to a very inefficient use, causing their rapid depletion from the culture medium and eventually the accumulation of lactate and ammonium ions in the medium. These products often have inhibiting effects for the cells, specially in the case of ammonium (Hansen and Emborg, 1994, Ozturk et al., 1992; Ryll et al., 1994). It has previously been demonstrated in chemostat cultures (Sanfeliu et al., 1997) that cells cultured in media with high glucose and glutamine concentrations exhibit much higher uptake rates than those strictly necessary for their metabolism. The solution to this problem has been addressed in many different ways. From the macroscopic point of view, which is at the cellular level, one of the findings to highlight is the definition of the extracellular conditions in the bioreactor where cell growth takes place, the fed-batch addition of glucose and glutamine to the medium (Ljunggren and Häggström, 1994; Xie and Wang, 1994; Bibila and Robinson, 1995; Zhou et al., 1995; Zhou et al., 1997) or the in situ removal of the inhibitory compounds (Matsumara and Nayve, 1995) are two possible strategies that generally require a high degree of instrumentation to be performed in an optimal way. From the microscopic point of view, which is at the cellular level, one of the findings to highlight is the modification of hybridoma and myeloma cells with the glutamine synthetase gene (Bibila and Robinson, 1995; Zhou et al., 1997; Paredes et al., 1999). In this way, cells can grow in medium with glutamine being replaced by glutamate, which is consumed in a more efficient way, producing a lower ammonium accumulation and allowing the extension of fed-batch cultures for a longer time. In this case it is interesting to point out that the glucose consumption and lactate accumulation is also reduced, showing how glutaminolysis and glycolysis pathways are interconnected in the metabolism of these cells. An intermediate possibility to improve cell growth patterns is to find alternative compounds for the cells to use as carbon and energy source, in theory without the need to undertake any genetic modification. The basic requirements for this substitution would be, first, that cells could grow in the new conditions, and second, that the metabolic uptake of the new compounds would be markedly reduced.
lower than glucose and glutamine uptake. In this way, it could be envisaged that the cells would use the main compounds of the medium more efficiently, generating a smaller amount of metabolic waste products, particularly lactate and ammonium, and therefore allowing the establishment of fed-batch or high-density perfusion cultures without inhibition limits.

The simultaneous substitution of both glucose and glutamine in the medium formulations has not been addressed previously, and only partial substitutions have been discussed, although in most cases not as a result of an analysis at the process level, linked to the metabolic patterns of the cells. Indeed, for some cells, particularly for hybridomas, it has been shown that this substitution is not possible (Wagner, 1997), at least not without genetic modification of the cells, for example, with the glutamine synthetase approach already discussed. For other cell lines, more studies have been reported. Wagner at al. (Wagner at al., 1991) showed that human tumor kidney cells could be grown on microcarriers, with slower cell death, using a mixture of glucose and galactose. Kurano et al. (Kurano et al., 1990), after discussing the inhibitory levels of ammonium and lactate, replaced glutamine by asparagine for the culture of CHO cells, obtaining a clear reduction in ammonium accumulation and the improvement of cell growth. These results were also confirmed by the work of Hansen and Emborg (Hansen and Emborg, 1994), Reitzer et al. (1979) in their very interesting discussion on the identification of glutamine as the major energy source for HeLa cells showed that cells can grow in a similar way on galactose or fructose as the main sugar in the medium instead of glucose, providing remarkably lower amounts of lactate. Marquis et al. (1996) provided data on the growth of a lymphoblastoid cell line on a medium with glucose replacement by galactose, with lower lactate production.

In addition to the changes in the primary metabolism that the substitution of glucose and glutamine would generate in the cells, it is also very interesting to study other metabolic modifications, for example, at the level of amino acids consumption or excretion, to generate hypotheses about the overall changes introduced in the cell behavior. In the present work, the substitution of glucose and glutamine in a low-protein medium for the culture of a Chinese hamster ovary (CHO) cell line producing t-PA in batch systems is analyzed from the point of view of the physiological changes observed in the cells and their internal metabolic redistribution. This substitution has been addressed by selecting a slowly metabolized sugar compound to replace glucose as carbon source. This compound should be a hexose sugar, as the cells used (a CHO cell line) do not perform gluconeogenesis activity. The entry of the sugar molecule into the glycolytic pathway should therefore be upstream of the metabolic step generating phosphogluconate, which in turn is essential to generate pentose molecules for the nucleic acids synthesis. With regards to glutamine, the objective of this work is to investigate its replacement by glutamate, as CHO cells do have the glutamine synthetase activity expressed constitutively. In addition to the fact that glutamate has only one amino group in its structure, the fact that glutamate is transported into the cell at a lower velocity should provide a more efficient use of this compound with lower generation of ammonium, because spontaneous decomposition would also then be minimized, with respect to the situation where glutamine is used.

### Material and Methods

**Cell Culture.** The cell line CHO TF 70R, kindly provided by Pharmacia & Upjohn (Stockholm, Sweden), has been manipulated genetically to produce t-PA. The cell cultures were carried out in spinner flasks (Techne) with a working volume of 125 mL or 250 mL and stirred at 50 rpm in a CO₂ incubator (Forma Scientific CO₂ incubator), at 37.0 °C, with 96% relative humidity in an atmosphere of 5% CO₂ in air.

**Culture Medium.** The basal medium used in all of the cultures was a proprietary serum-free and low-protein medium denominated BIOPRO1, kindly provided by Bio Whitaker Europe (Verviers, Belgium). BIOPRO1 was supplemented with vitamins (Sigma), lipids and cholesterol ( GibcoBRL), proline, serine, and aspartic acid (Sigma). This medium was also supplemented with 20 mM glucose, fructose, mannose, or galactose and 7 mM glutamine or glutamic acid in the different experiments, as indicated in Figures 1 and 3. The culture of the cells in the different media was carried out without previous adaptation. Cells used for innoculum (0.18–0.20 × 10⁶ cells/mL) were centrifuged to remove the culture medium, particularly to ensure that neither remaining glucose nor glutamine from inoculum preparation medium was present. This step was repeated 3–5 times, keeping the cells subcultured in the corresponding carbon and nitrogen source combination under study, before the realization of the experiment. In those passages the growth of the cells was very reproducible.

**Analytical Methods. Cell Number.** Cells were counted using a hemacytometer (Neubauer improved, Brand). Cell viability was determined by the trypan blue exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). After cell counting, the remainder of each sample was centrifuged (5000g, 1 min) to remove the cells, and the supernatant was frozen for further analysis.

**Metabolite Determinations.** Glucose and lactate concentrations were determined with a YSI 2700 automated glucose and l-lactate analyzer. Ammonium concentration was determined by a flow injection analysis system as previously described (Campmajó, 1994). Amino acids concentrations were measured by HPLC (Hewlett-Packard 1090) using a reversed-phase column (amino-exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). After cell counting, the remainder of each sample was centrifuged (5000g, 1 min) to remove the cells, and the supernatant was frozen for further analysis.

**Specific Rate Calculations.** The specific growth rate ($\mu$) in the exponential growth phase was calculated by linear regression of the logarithm of the concentration of viable cells, $X_v$, as a function of time, $t$, (eq 1)

$$X_v = X_{v0} \exp(\mu t)$$  \hspace{1cm} (1)

In the above expression, $X_{v0}$ is the initial cells number ($10^6$ cell mL⁻¹). The specific consumption/production rates, $q_i$ in nmol h⁻¹ 10⁻⁶ cells, were calculated using the following expressions

$$S = S_0 + \frac{q_i 10^{-3}X_{v0}}{\mu} [\exp(\mu t) - 1]$$ \hspace{1cm} (2)

where $S$ is the concentration of the different compounds
investigated (carbon and nitrogen sources, and products) and $S_0$ refers to its initial concentration (mM).

In the case of glutamine, this spontaneously decomposes, releasing ammonia following first-order kinetics. Its rate constant, $k_d$, has been evaluated in the experimental conditions used in this work, obtaining a value of $2.01 \times 10^{-3}$ h$^{-1}$. Thus equations for glutamine and ammonium were modified conveniently to take into account this fact:

$$\text{Gln} = \text{Gln}_0 \exp(-k_d t) + \frac{q_{\text{Gln}} \cdot 10^{-3} X_v}{k_d + \mu} \exp(-k_d t)$$

$$\text{NH}_4^+ = \text{NH}_4^+ \exp(-k_d t) + \frac{q_{\text{Gln}} \cdot 10^{-3} X_v}{k_d + \mu} \exp(-k_d t)$$

**Results and Discussion**

**Substitution of Glucose by a Hexose.** In a first series of batch experiments, the glucose substitution in the formulation of the culture medium was investigated, with glutamine maintained in all cases as nitrogen source. Three different compounds were tested: fructose, galactose, and mannose. Glucose was used as the control system. The results are presented in Figure 1. It can be observed (Figure 1a) that the maximal cell concentration achieved for glucose and mannose is similar, reaching a level of $1.2 \times 10^6$ cells/mL, whereas for galactose and fructose it is lower, $0.8 \times 10^6$ and $0.5 \times 10^6$ cells/mL, respectively. The specific growth rate on glucose and mannose was almost the same, as reflected in Table 1, and higher than for galactose and fructose. With respect to the consumption of the carbon source (Figure 1c), two groups of compounds can be distinguished, with marked differences: glucose and mannose are consumed at a high rate, whereas fructose and galactose are consumed much more slowly. The specific consumption rates are provided in Table 1, and it should be highlighted that the values observed for the consumption of fructose and galactose are around 25% of that for glucose. As a consequence of these different consumption rates, the generation of lactate also differs greatly. For glucose and mannose, the high consumption rate leads to high lactate generation, and lactate concentration reaches levels of about 16 mM (Figure 1d), with high values for the specific production rate (see Table 1). On the other hand, when cells are grown on fructose and galactose, the lactate production decreases drastically, and final lactate concentrations are below 1 mM. The specific lactate production rates being about 22% of the values corresponding to glucose. When the glutamine consumption is analyzed (Figure 1e) a similar profile is observed for all of the cultures; however, taking into account the different level of cells generated, it can be calculated that the specific consumption of glutamine is notably higher when galactose and fructose are used, as reflected in Table 1. It can be observed that in the case of the medium formulation with galactose, an abrupt cell concentration decline occurs at the point when glutamine is exhausted. These profiles are linked to those corresponding to the ammonium ion generation in the medium (Figure 1f), which are higher for the compounds providing a higher specific consumption of glutamine (fructose and galactose), reaching levels of 13 mM for ammonium, and lower when glucose or mannose are used, reaching 9 mM for ammonium. Finally it should be mentioned that the profile of the t-PA concentration follows that of the cell growth (Figure 1b), and therefore a higher t-PA level is obtained when a higher cell concentration is generated.

The analysis of the previous results from the metabolic approach can be done at different levels. The scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells is presented in Figure 2. It can be observed that mannose enters into the glycolytic pathway at the level of fructose-6-P, whereas galactose does so at the level of glucose-6-P. Nevertheless, mannose is consumed at a much faster speed than fructose, with a rate similar to that for glucose.

The observation of the experimental data suggests some hypotheses with respect to the transport of these compounds into the cells. Mannose and glucose seem to be transported at the same velocity into the cell, either through the same transporter or a different one. On the other hand, fructose is probably transported more slowly into the cells, as a result of a lower affinity of the fructose transporter. With respect to galactose, in theory one might expect to find a consumption rate similar to glucose, as both sugar molecules are incorporated into the glycolysis at the level of glucose-6-P. However, the observed rate is considerably slower, and again this fact could be explained by a low transport rate of galactose into the cells. Indeed, it has been previously reported that, for several mammalian cell lines (Plagemann et al., 1981) galactose is taken up into the cells through the same transporter as glucose but with an affinity which is eight times lower. A second possibility that could explain the different consumption rate of the compounds studied is a lower affinity of the enzyme hexokinase for galactose and fructose (Wagner, 1997).

For the quickly metabolized sugars, glucose and mannose, the cell converts most of them into pyruvate through the glycolytic pathway, and probably a small percentage is cycled through the pentose phosphate cycle to obtain the necessary molecules to build compounds essential for the cell viability. It can also be observed from the experimental results that a high percentage of pyruvate is metabolized to lactate. The explanation for this fact may be found in the need for the cell to balance its redox potential (Paredes et al., 1998). It should also be mentioned that it has been previously demonstrated that this pattern has been observed in other cell lines, independently of the oxygen level in the culture system (Sanfelu et al., 1996), and in consequence it cannot be attributed to a lack of oxygen.

It can be observed that for the sugars presenting a lower glycolytic rate, fructose and galactose, glutamine is consumed at a higher specific rate (see Table 1) and that for similar total consumption values (see Figure 1e), a higher ammonium yield is obtained (see Figure 1f). The main metabolic intermediate through which glycolysis and gluconolysis pathways are connected is pyruvate, which also is the key intermediate compound between glycolysis and the tricarboxylic acids cycle. Because of the low specific consumption rate for fructose and galactose, it should be assumed that, in this situation, most of the carbon is diverted to the pentoses synthesis and therefore only a small amount of pyruvate is generated (Reitzer et al., 1979). This fact leads to two consequences: first, the amount of lactate generated in the culture medium is minimal; second, the depletion of pyruvate would stop the TCA cycle, unless the cell could provide more pyruvate from gluconolysis. One possible
reaction that the cell can activate to compensate the low generation of pyruvate is that catalyzed by the malic enzyme (Sauer et al., 1980; Zielke et al., 1980), enabling the transformation of malate (that is then exported from TCA) into pyruvate (see Figure 2).

It is also of interest to discuss the differences encountered with respect to alanine production. Indeed, as already mentioned, although glutamine is consumed in almost the same total quantity for the four sugars studied, ammonium is produced in higher amounts when the sugar is consumed slowly (i.e., fructose and galactose), and at the same time alanine is produced in a much lower quantity than for the culture on glucose or mannose (level of alanine at 180 h of culture, 0.7 mM for galactose and fructose and 3.0 mM for glucose and mannose). Alanine is normally obtained in cell culture as a product of the transamination reaction between pyruvate and glutamate. The reaction also produces α-ketoglutarate, and generally this is the most common way to obtain this compound in cells that are proliferating rapidly, simultaneously reducing the ammonium generation (Matsuno et al., 1994). The low alanine production that is observed for the slowly metabolizable sugars should probably be linked to the low availability of pyruvate in the cell due to the slow glycolysis. In that situation, most of the pyruvate provided by the low glycolytic flux must be incorporated by the cells into the TCA cycle, and the transamination to alanine is then less favored. Alternatively, the generation of α-ketoglutarate is provided by the action of the enzyme glutamate dehydrogenase.

Finally, it should be pointed out that glycine excretion is also changing with the carbon source used. For sugars rapidly metabolized, a lower glycine production is observed when compared to slowly metabolized sugars (level of glycine at 180 h of culture, 1.2 mM and 2.5 mM, respectively). Glycine is related to pyruvate metabolism.

**Replacement of Glutamine by Glutamate.** In the second series of experiments of this work, the same approach was followed to substitute glutamine in the culture medium, with the main objective of obtaining a more efficient use of the nitrogen source, with a lower release of ammonium ions. In this case, taking into account the previous results published in this respect (Hansen and Emborg, 1994, Ozurk et al., 1992; Ryll et al., 1994) and considering the glutamine metabolism (see Figure 2), glutamate was selected directly as the compound to be used, for three main advantages: first, glutamate does not suffer spontaneous decomposition as does glutamine (ammonium ions will not be generated as a result); second, glutamine can be obtained directly from glutamate by the CHO cells, as they exhibit glutamine synthetase activity; and third, glutamate has only one amino group. With respect to the carbon source used in this second series of experiments, in view of the results presented in the previous section, it was decided to select galactose, as a slowly metabolizable sugar, and glucose as the control system. The results of the four experiments carried out, using glutamine and glutamate as nitrogen sources and glucose and galactose as carbon sources, are presented in Figure 3.

In Figure 3a it can be observed that the final cell density attained growing on glucose–glutamate is the highest, being more than two times the concentration attained with the combination galactose–glutamate (1.8 × 10^6 and 0.8 × 10^6 cells/mL respectively), and also notably higher that the final cell concentration for the

**Figure 1.** Results of the batch cultures of the CHO cells, using glutamine (7 mM) and four different sugar compounds in the medium formulation: 20 mM fructose ( ), 20 mM galactose ( ), 20 mM mannose ( ), and 20 mM glucose ( ) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

| Table 1. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Cell Line Growing on Different Carbon Sources |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | glucose         | fructose        | mannose         | galactose       |
| μ (h⁻¹)          | 0.0227          | 0.0109          | 0.0220          | 0.0153          |
| qC              | -176.1          | -48.7           | -203.7          | -48.0           |
| qLac            | 336.2           | 78.8            | 364.7           | 61.9            |
| qGln            | -65.9           | -103.3          | -79.9           | -115.0          |
| qNH₄            | 48.7            | 85.2            | 52.8            | 78.5            |
control culture, glucose—glutamine. The cell profiles for the growth on galactose—glutamate and galactose—glutamine are quite similar, with the main difference being that the abrupt drop in the cell concentration observed for galactose—glutamine is not observed for galactose—glutamate, probably because of the very much lower ammonium level generated, as will be discussed below, and also because glutamine is exhausted more rapidly in the first case, that of galactose—glutamate formulation. Regarding t-PA concentration profiles, as in the previous section, there is a clear proportionality between cell growth and product formation.

Figure 3c reflects the profiles for the consumption of the carbon source. It can be observed that galactose is consumed more slowly than glucose, as discussed in the previous section, and also that in the presence of glutamate, the consumption of the carbon source is always lower that in the presence of glutamine, both for glucose and galactose, as reflected in Table 2. As previously mentioned this fact highlight the relationship existing between glycolysis and glutaminolysis. When the glutaminolysis rate is decreased by the replacement of glutamine by glutamate, the cells possibly have a lower need for pyruvate to be incorporated into the TCA cycle, and therefore the glycolysis rate is also reduced. This behavior is also found in other cell lines; the most evident example is for hybridoma cells modified genetically with the introduction of the glutamine synthetase gene. These transformed cells can then be grown on medium with glutamine being replaced by glutamate, and the pattern observed is a lower glutamate consumption rate, together with a decrease of about 50% in the consumption of glucose (Bibila and Robinson, 1995; Paredes et al., 1999).

With respect to the generation of lactate, Figure 3d shows that it is minimal for the media formulated with galactose, whereas for the media containing glucose, a clear difference can be observed between the control culture, glucose—glutamine (16 mM of lactate) and the experiment with glucose—glutamate (7 mM), clearly as a consequence of the lower consumption rate for glucose when glutamine is replaced by glutamate.

The data for the consumption of the nitrogen source are presented in Figure 1e. The trends for the consumption of glutamine and glutamate can clearly be distinguished, the later being a compound more slowly metabolized by the cells. The specific uptake rate values for the different combinations of media formulation are given in Table 2. The most relevant consequence of this different uptake profile is the ammonium generation. Indeed, it is markedly lower when glutamate is used instead of glutamine. It is also interesting to appreciate that the carbon source also affects, although to a minor extent, the ammonium generation, the general trend being that for any of the two nitrogen sources, ammonium concentration always reaches higher values for galactose than for glucose. Again, this fact also reflects the flexibility of the metabolic network. When the cells are cultured in galactose, the low uptake rate is most probably the limiting factor for cell metabolism, and it is compensated by an increase of the nitrogen source uptake (either glutamate or glutamine), generating a higher amount of ammonium. When glutamate is used in the medium, the alanine production decreases for any of the sugars tested (level of alanine at 180 h, glucose—glutamate culture, 0.7 mM; and galactose—glutamate culture, 0.1 mM) probably as a consequence of a lower availability of the key intermediate, pyruvate, which is transaminated to alanine and α-ketoglutarate in a much lower extent. On the other hand, the glycine production reaches similar levels to those obtained when glutamine is used, independently of the carbon source used (glucose or galactose).

Figure 2. Scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells.

Conclusions

The results presented demonstrate the possibility of regulating the metabolic pattern of CHO cells by substitution of the most commonly used main compounds in the medium formulation, glucose and glutamine, by other compounds that are taken up at significantly lower rates. As most of these compounds are incorporated to the central metabolism, it is most probable that transport rate into the cell is the major step controlling this process; or alternatively it could also be due to the different affinity of the hexokinase enzyme for the different
compounds. From the different possibilities studied, the combination of galactose and glutamate allows the maintenance of cells at an optimal situation with regard to minimal consumption of nutrients and minimal generation of lactate and ammonium, although a lower cell growth is obtained. Alternatively, glucose and glutamate allow the obtaining of maximized cell growth, significantly higher than the control culture with glucose and glutamine, with very low ammonium accumulation and reduced lactate levels. These conditions offer the possibility for optimization of fed-batch cultures, as it will be possible to expand the time span of the cultures because the limiting conditions in terms of osmolarity and toxic compound accumulation will occur later than in the conventional medium formulation with glucose and glutamine.

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**References and Notes**


**Figure 3.** Results of the batch cultures of the CHO cells, using glutamine (7 mM) or glutamate (7 mM) as nitrogen sources and galactose (20 mM) or glucose (20 mM) as carbon sources in the medium formulation: galactose—glutamine (△), galactose—glutamate (○), glucose—glutamate (□), and glucose—glutamine (●) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

**Table 2. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Line Growing on Different Nitrogen Sources**

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